THE UNIVERSITY *of* EDINBURGH

Oestrogen metabolism and action in epithelial ovarian cancer

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Declaration

It is hereby declared that I am the author of this thesis and that I did all the work described herein, unless otherwise specified. Contributions made by colleagues have been duly acknowledged by the means of references. This work has not been submitted for any other degree or professional qualification.

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Abstract

Ovarian cancer is the most fatal of all gynecological malignancies. Epithelial ovarian cancer (EOC) accounts for about 90% of malignant ovarian tumours and is thought to originate mostly from ovarian surface epithelium (OSE) cells. Epidemiological data suggest that hormone replacement treatment (HRT) users have a higher risk of ovarian cancer, which is related to the use of oestrogen-only HRT. In addition, EOC is oestrogen responsive. This thesis reveals the capacity for production and metabolism of oestrogen in normal OSE and malignant primary EOC cells, and describes the action of oestrogen in the development of EOC at three levels.

First, the expression of the genes encoding oestrogen production and metabolism and oestrogen receptor (ER) was investigated in OSE and EOC cells at RNA and protein levels. Immunohistochemistry revealed that steroid sulphatase (STS), oestrogen sulfotransferase (EST), 17βhydroxysteroid dehydrogenase (17βHSD) 2 and 17βHSD5 proteins were present in pre-menopausal, post-menopausal and inclusion cystic OSE as well as EOC cells. Taqman qRT-PCR revealed STS, EST, 17βHSD1, 17βHSD2, 17βHSD5, ERα, ERβ and oestrone sulphate (E_1S) transporters such as organic anion transporting polypeptide (OATP)-B, OATP-D and OATP-E mRNAs were expressed in pre-menopausal OSE and EOC at different levels. When basal mRNA levels were compared among untreated samples of pre-menopausal OSE and EOC, EST mRNA expression was significantly higher in the OSE compared to EOC cells ($P<0.05$) while OATP-B mRNA level was the opposite ($P<0.05$). Radiometric enzyme activity assays demonstrated different metabolism patterns of E_1S and oestrone (E_1) between normal and malignant cells, indicating overall activities of STS and 17βHSD1 or 17βHSD5 to be higher than the overall activities of EST and 17βHSD2 in cancer cells while enzyme activities in OSE cells were opposite to this. Second, the impact of inflammation on oestrogen production, metabolism and action was compared in OSE and EOC cells by testing the response of target genes to a panel of pro-inflammatory cytokines. The data revealed that in OSE cells, EST ($P<0.01$) and 17βHSD2 ($P<0.001$) mRNAs were decreased while ERα mRNA ($P<0.001$) was increased by IL-1α. In addition, EST mRNA was inhibited by IL-4 ($P<0.05$). In SKOV-3 (EOC cell line) cells, IL-1α stimulated STS mRNA ($P<0.001$)

and enzyme activity ($P<0.05$). Moreover, IL-4 inhibited ($P<0.05$) while IL-8 and IL-10 enhanced ($P<0.01$) ERα mRNA levels. Finally, the effect of oestrogenic components of HRT medication (equilin and equilin-sulphate) on the expression of cancer-associated genes was compared to that of 17β-oestradiol (E_2) in PEO-1 (an oestrogen-responsive EOC cell line) cells. Expression of the oestrogen-responsive genes FN1 and IGFBP3 mRNA expression was similarly inhibited by E_2 and equilin ($P<0.05$) as well as E_1S and sodium equilin-sulphate ($P<0.05$).

In conclusion, this thesis presents evidence that intracrine oestrogen formation and metabolism differs between OSE and EOC cells, such that E_2 formation is inhibited in normal OSE but is promoted in EOC. Inflammatory cytokines also influence the local production of E_2 by regulating genes encoding oestrogen production and metabolism and receptors. Finally, local HRT metabolites can regulate cancer-associated gene expression in EOC. Together, these data suggest a role for local oestrogen production and action in inflammation-associated development of EOC. Conversely, differential regulation of the same parameters in OSE cells from pre-menopausal women minimizes oestrogen formation and 'protects' against the promotion of EOC.

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List of abbreviations

A

A: androstenedione

AF: activation function

AI: aromatase inhibitor

AJCC: American joint committee of cancer

AKR: aldoketoreductase

ANOVA: analysis of variance

AR: androgen receptor

B

bp: base pair

BRCA: breast cancer

BSA: bovine albumin serum

C

CA125: cancer antigen 125

cAMP: 3'-5'-cyclic adenosine monophosphate

cDNA: complementary DNA

CEE: conjugated equine oestrogen

D

DAB: 3,3-diaminobenzidine

DBD: DNA binding domain

DiagLapar: diagnostic laparoscopy

DHEA: dehydroepiandrosterone

DHEAS: dehydroepiandrosterone sulphate

DHT: dihydrotestosterone

DPBS: Dulbecco's phosphate buffer saline

DNA: deoxyribonucleic acid

dNTP: deoxynucleotide triphosphate

E

ECM: extracellular matrix

EGF: epidermal growth factor

EOC: epithelial ovarian cancer

ER: oestrogen receptor

ERE: oestrogen response element

ERT: oestrogen replacement therapy

EST: oestrogen sulfotransferase

E_2: 17β-oestradiol

E_1S: oestrone sulphate

E_3: oestriol

Eq: equilin

F

FBS: fetal bovine serum

FIGO: International Federation of Obstetrics and Gynaecology

FGF: fibroblast growth factor

FN1: fibronectin

FSH: follicle-stimulating hormone

FW: Forward

G

G: grade

GFP: green fluorescent protein

Glu: Glusulase

GPR30: G protein-coupled receptor 30

GM-CSF: granulocyte macrophage-colony stimulating factor

GnRH: gonadotrophin releasing hormone

GR: glucocorticoid receptor

H

hCG: human chorionic gonadotrophin

HIF: hypoxia-inducible factor

HMB: heavy menstruation bleeding

HRP: horseradish peroxidase

HRT: hormone replacement therapy

HSD: hydroxysteroid dehydrogenase

I

IC: inclusion cyst

IFNR: interferon receptor

IGF: insulin growth factor

IGF-IR: IGF-I receptor

IGF-IIR: IGF-II receptor

IGFBP3: insulin-like growth factor binding protein 3

IHC: immunohistochemistry

IL: interleukin

IL-1R: IL-1 receptor

IL-1R1: IL-1 receptor type 1

IL-1R2: IL-1 receptor type 2

IL-1RA: interleukin-1 receptor antagonist

IL-4R: IL-4 receptor

IR: insulin receptor

IVF: *in vitro* fertilization

L

LBD: ligand binding domain

LH: luteinising hormone

LOX: lysyl oxidase

LREC: Lothian Research Ethical Committee

M

M: mol/L

MA: megestrol acetate

MAPK: mitogen activated protein kinase

GM-CSF: granulocyte-macrophage-colony-stimulating factor

MMP: matrix metalloproteinase

MPA: medroxyprogesterone acetate

mRNA: messenger RNA

N

NaE_1S: sodium oestrone sulphate

NaEqS: sodium equilin sulphate

NADH: reduced form of nicotinamide adenine dinucleotide

NADPH: reduced form of nicotinamide adenine dinucleotide phosphate

NBF: neutral buffered formalin

NF-κB: nuclear factor-kappa B

NO: nitric oxide

nM: nanomolar

O

OAT: organic anion transporter

OATP: organic anion transporting polypeptide

OSE: ovarian surface epithelium

P

PBSO: prophylactic bilateral salpingo-oophorectomy

PCOS: polycystic ovarian syndrome

PCR: polymerase chain reaction

PDGF: platelet-derived growth factor

PG: prostaglandin

PGE2: prostaglandin E2

PID: pelvic inflammatory disease

PI-3K: phosphatidyl-inositol-3 kinase

PKA: protein kinase A

PA: plasminogen activator

pM: picomolar

PPAR: proliferation-activated receptor

PR: progesterone receptor

Prog/P4: progesterone

PST: phenol sulfotransferase

Q

qPCR: quantitative PCR

R

R: receptor

RNA: ribonucleic acid

RT: reverse transcription

RT-ve: RT negative

RV: reverse

S

SDR: short-chain alcohol dehydrogenase/reductase

SEM: standard error of the mean

SERM: selective oestrogen receptor modulator

SLC: solute carrier

ST: sulfotransferase

STAT: signal transducers and activators of transcription protein

STS: steroid sulphatase

SULT: sulfotransferase

T

TAH: total abdominal hysterectomy

TAHBSO: total abdominal hysterectomy and bilateral salpingo-oophorectomy

TBS: tris buffered saline

TGF-β1: transforming growth factor-β1

Th-2: T-helper 2

TLC: thin layer chromatography

TLR: toll-like receptor

TNF: tumour necrosis factor

U

UNG: Uracil-N-glycosylase

uPA: urokinase plasminogen activator

W

WHO: World’s Health Organisation

Misc

µM: micromolar

Chapter 1

General introduction and literature review

The aim of this thesis is to demonstrate that the surface of the human ovary, the ovarian surface epithelium (OSE), contains the machinery to produce, activate and respond to hormonal oestrogens. The translational importance of this is that OSE is the site of development of potentially lethal, oestrogen-responsive disease in the form of epithelial ovarian cancer (EOC). Understanding factors controlling oestrogen metabolism in the OSE may therefore unlock new therapeutic options. This literature review presents a background to the experimental studies described in later sections of the thesis.

1.1 The human ovary

1.1.1 Ovarian histology

The adult ovary is the source of oocytes and also responsible for much of the production and action of sex hormones. They are located in pairs in the abdominal cavity on either side of the uterus as part of the female reproductive tract. The adult ovary comprises three zones of stroma: the outer cortex, the inner medulla and the hilus, containing follicles at various stages of development, corpora lutea and corpora albicans. The tunica albuginea is a tough fibrous layer that surrounds the outer cortex. External to this is OSE, which is loosely attached to a thin basal lamina on the surface of the tunica albuginea (Fig. 1.1)

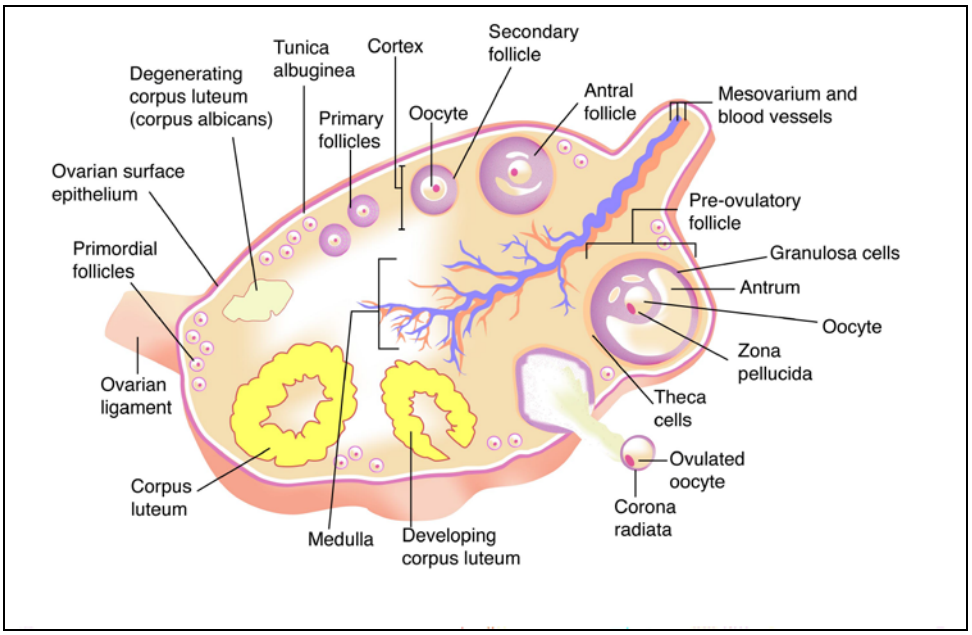

Figure 1.1: The human ovary. Follicles at various stages of development with corpus luteum and corpus albicans are located in cortical region. The medullary region contains arteries and veins. The OSE is separated from tunica albuginea by a basal lamina. Picture courtesy of Dr Chris Harlow.

The OSE comprises a single layer of mesothelial squamous-to-cuboidal cells covering the entire surface of the ovary and continuous with the peritoneal mesothelium. Normal OSE is developmentally a mesothelium with both epithelial and mesenchymal characteristics. In response to stimuli that initiate a regenerative (repair) response, such as ovulatory rupture *in vivo* or explantation into culture, OSE cells assume phenotypic characteristics of stromal cells. Additionally, OSE acquires complex epithelial characteristics of the Mullerian duct-derived epithelia, when it undergoes metaplasia, benign tumour formation, and neoplastic progression (Auersperg, Wong et al. 2001). Normal OSE does not have known tissue-specific differentiation markers. However, it has classical epithelial features, which include desmosomes, tight junctions and keratin (van Niekerk, Boerman et al. 1991) but rarely expresses E-cadherin and cancer Antigen 125 (CA125) (Kabawat, Bast et al. 1983). *In situ*, it expresses mucin and 17β hydroxysteroid dehydrogenases (17βHSDs) (Nicosia and Johnson 1984) and it lacks CA125, which distinguish it from extra-ovarian mesothelium. OSE provides the external cellular barrier of the ovary,

transports materials to and from the peritoneal cavity and takes part in the cyclical ovulatory rupture and repair (Auersperg, Wong et al. 2001). In the last decade, many studies have implicated OSE as the main source of ovarian cancer, and interest in this compartment of the ovary has risen sharply. The role of OSE in the initiation and development of ovarian cancer will be discussed later.

Although OSE is not thought to be a major site of steroid hormone synthesis, it expresses genes encoding steroid hormone biosynthesis and nuclear hormone receptors suggesting a role of these cells in ovarian function. An oligonucleotide microarray analysis revealed mRNA expression for key enzymes required to synthesis *de novo* steroids in the C21-pregnane and C19-androstane series and the expression of dehydrogenase, hydroxylase, conjugation and deconjugation enzymes. The presence of these enzymes suggested the possibility of intracrine steroid generation in the cultured OSE cells (Rae, Niven et al. 2004b). Notably, the Rae study showed the presence of transcripts for steroid sulphatase (STS) and 17βHSD1 enzymes that are involved in oestrogen biosynthesis, highlighting the potential for production of biologically active oestrogen from inactive precursors *in situ*. Therefore, it is interesting that OSE cells can synthesize steroid precursors from cholesterol and also use precursors produced by extra-ovarian or adjacent ovarian cells to generate bioactive steroid. In addition, the expression of gonadotrophin-releasing hormone receptor (GnRHR) (Kang, Cheng et al. 2000), follicle-stimulating hormone receptor (FSHR) (Choi, Kang et al. 2002) and luteinizing hormone receptor (LHR) (Kuroda, Mandai et al. 2001), androgen receptor (AR), progesterone receptor (PR), oestrogen receptor (ER) including both ERα and ERβ (Lau, Mok et al. 1999; Edmondson, Monaghan et al. 2002) and glucocorticoid receptor-α (GRα) (Rae, Niven et al. 2004a) in OSE indicates that OSE is a steroid target. Moreover, thyroid hormone receptor, retinoic acid receptor and peroxisome proliferation-activated receptor (PPAR) are also expressed in OSE cells (Rae, Niven et al. 2004b). Thus the OSE is a site of steroid production and a target of hormone action, consistent with the idea that hormone signalling in the OSE can critically influence the initiation and development of ovarian cancer.

1.1.2 Ovulation and inflammation

1.1.2.1 Ovulation process

Ovulation is the release of a fertilizable oocyte from the adult ovary. This tightly controlled process occurs on a monthly basis and lasts as much as 30-36h in human. During folliculogenesis, by the late follicular phase of the menstrual cycle only one follicle becomes mature. The LH surge initiates the ovulatory process and the apex of the mature follicle protrudes progressively above the surface of the ovary, while the follicle wall gradually becomes thinner to form a stigma from where the follicle ruptures. Following proteolytic degradation of follicular cells and OSE layer at the apex of the ovulation site, the follicle wall bursts and the oocyte is released into the peritoneal cavity. Ovulation is complete when the egg-bearing cumulus mass is expelled from the ovary (Espey 1999).

1.1.2.2 Ovulation and inflammation

30 years ago, Espey first proposed the hypothesis that ovulation was comparable to an acute inflammatory reaction, based on similar physical and biochemical changes occurring during ovulation and acute inflammation (Espey 1980). The inflammatory reaction causes an increase in vasodilatation, hyperemia, exudation, oedema, collagenolysis, cell proliferation, tissue remodelling, and other common changes in inflamed tissue (Espey 1994). Like their role in other inflammatory events, leukocytes appear to be critical to ovulation. There is a marked increase in both neutrophil and macrophage density in the theca layer of the ovulatory follicles just prior to ovulation and leukocyte-derived inflammatory factors including prostaglandins (PGs), bradykinin, histamine, platelet activating factor and various cytokines are potential mediators of the ovulatory process (Espey 1994). For example, histamine released by mast cells is responsible for increased vascular permeability observed during ovulation (Abisogun, Daphna-Iken et al. 1988). Ovarian follicular wall collapse is mediated by proteolytic enzymes such as plasminogen activator (PA) and elastase that are mainly released by neutrophils and macrophages (Espey 1994).

Following ovulation associated tissue damage at the site of oocyte expulsion, anti-inflammatory repair and reorganization of the site is necessary to form a corpus luteum. During the repair process, nitric oxide (NO) released by invading leukocytes facilitates vasodilatation and secretion of interleukin (IL)-1 for tissue remodelling (Fleming, Beaugie et al. 2006). Epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) are released from platelets, and pro-inflammatory cytokines and prostanoids stimulate OSE growth (Auersperg, Wong et al. 2001). Additionally, infiltrating macrophages produce tumour necrosis factor (TNF-α), which also facilitates proliferation of OSE (Auersperg, Wong et al. 2001).

1.1.2.3 Cytokines involved in ovulation

As mentioned above, ovulation is an acute inflammatory process involving leukocytic invasion and local production of pro-inflammatory cytokines. The ovulatory follicle becomes surrounded by cytokines such as IL-1 and TNF-α secreted by neutrophils, macrophages, and lymphocytes that have migrated to the site of rupture (Brannstrom and Norman 1993). Several cytokines such as IL-1, IL-2 (Wang and Norman 1992), TNF-α (Wang, Brannstrom et al. 1992) and granulocyte-macrophage-colony-stimulating factor (GM-CSF) (Jasper, Brannstrom et al. 1996) have been demonstrated in human pre-ovulatory follicular fluid obtained during follicular aspiration at oocyte recovery for *in vitro* fertilization (IVF). IL-4 (Hashii, Fujiwara et al. 1998) and IL-8 (Zhao, Rong et al. 1995; Buscher, Chen et al. 1999) are also found in the follicular fluid of the peri-ovulatory follicle, supporting roles for each of these inflammatory mediators in ovulation as well.

There is also experimental evidence that cytokines facilitate the process of ovulation. For example, IL-l and TNF-α promote ovulation in combination with LH in the perfused ovary (Brannstrom, Wang et al. 1993; Brannstrom, Bonello et al. 1995). In addition, they enhance the production of PGs in the intact isolated organ or from isolated follicles or ovarian cells (Brannstrom and Norman 1993). Cytokines also promote the post-ovulatory healing process. Thus, cytokines are important

determinants of ovarian function, including ovulation and formation of the corpus luteum.

1.1.3 Ovulation, inflammation and ovarian cancer

Given that ovulation resembles an inflammatory episode, involving secretion of immune mediators that impact the local cellular milieu, it is therefore of interest that epidemiological studies have identified ovulation and ovulation-associated hormones as risk factors in epithelial ovarian cancer (EOC). Thus if ovulation occurs relatively frequently, or at an artificially higher rate, or for longer periods during life (e.g. nulliparity, use of ovulation-inducing drugs, early age at menarche and late age at menopause) ovarian cancer risk increases (Franceschi, La Vecchia et al. 1991; Whittemore, Harris et al. 1992). Conversely, there is a decreased risk of developing EOC with pregnancy or oral contraceptive pill use; conditions which decrease ovulation (Whittemore, Harris et al. 1992; Adami, Hsieh et al. 1994; Risch, Marrett et al. 1994; Modan, Hartge et al. 2001; Titus-Ernstoff, Perez et al. 2001; Riman, Dickman et al. 2002; Riman, Nilsson et al. 2004). When the total lifetime ovulation number is calculated for women who have EOC and for those that do not, there is a significant correlation between high total ovulation number and the occurrence of ovarian cancer (Purdie, Bain et al. 2003). On the other hand, there have been many epidemiological studies demonstrating exposure to inflammatory factors such as talc or asbestos particles are associated with higher EOC risk (Purdie, Green et al. 1995; Ness and Cottreau 1999). Furthermore, endometriosis and pelvic inflammatory disease (PID), which cause local pelvic inflammation, are also EOC risk factors (Shu, Brinton et al. 1989; Brinton, Gridley et al. 1997). Conversely, hysterectomy and tubal ligation, which may decrease the likelihood that OSE will be exposed to environmental inflammatory stimuli, act as protective factors. Additionally, the risk of EOC in women who use low dose aspirin, acetaminophen or non-steroidal anti-inflammatory agents consistently for at least 6 months is reduced (Akhmedkhanov, Toniolo et al. 2001; Altinoz and Korkmaz 2004). Thus taken in combination, this evidence supports the idea that inflammatory events triggered either by ovulation or by extra-ovarian factors may impact upon OSE integrity, thereby initiating or promoting EOC (Ness, Grisso et al. 2000)

The role of inflammation in the development of cancer was first hypothesized in 1863 by Rudolf Virchow who recognized the infiltration of leukocytes into tumour tissues, suggesting initiation of tumour progression at sites of chronic inflammation (Balkwill and Mantovani 2001). A series of studies have been performed to investigate the mechanisms through which inflammation can promote the development of EOC. Firstly, ovulation may trigger cellular events that result in carcinogenesis. Repeated damage caused by ovulation and OSE proliferation to repair the wound, places a strain on the OSE, increasing the chance of errors occurring during DNA replication (Fathalla 1971). In women, mutations of the p53 tumour suppressor gene that are thought to result from spontaneous errors of deoxyribonucleic acid **(**DNA) synthesis during cell proliferation were associated with an increased number of lifetime ovulations (Schiffenbauer, Abramovitch et al. 1997). Moreover, inclusion cysts are formed as OSE become entrapped in the ovarian wound created during ovulation. OSE cells lining inclusion cysts are less plastic than the relatively pluripotent normal OSE, and more likely to proceed to ovarian carcinogenesis (Auersperg, Maines-Bandiera et al. 1997). Furthermore, inflammation produces toxic oxidants that can give rise to direct damage to DNA and proteins and may play a direct role in carcinogenesis (Dreher and Junod 1996). Also, chronic inflammation is associated with increased cell division, which can cause the potential for replication errors following DNA repair; aberrant DNA repair, especially at main regulatory sites, may increase the risk of mutagenesis (Ames, Gold et al. 1995). Moreover, cytokines, growth factors and prostaglandins involved in the inflammatory process may play an important role in ovarian mutagenesis and development. For example, the pro-inflammatory cytokine TNF-α, which has a role in the natural ovulation process, promotes EOC (Murdoch, Colgin et al. 1997). Its possible involvement in OSE neoplastic transformation is through regulating genes participating in membrane degradation, such as urokinase plasminogen activator (uPA) and matrix metalloproteinase (MMP)-9, prior to and after ovulation (Murdoch and Lund 1999; Yang, Godwin et al. 2004). More evidence for the positive link between inflammation and EOC comes from studies with IL-1α, which will be discussed in the next section.

In conclusion, inflammation-associated events affect the functionality of ovarian cells prior to and after ovulation, and inflammation is intimately involved with tumour initiation, progression and invasion. Taken together, this suggests a need to understand the physiological mechanisms of inflammatory and anti-inflammatory processes in the ovarian cell surface, as well as in ovarian cancer as a basis for developing novel molecular markers for diagnosing and treating ovarian cancer (Rae and Hillier 2005).

1.1.4 Interleukin system

1.1.4.1 IL-1 system

In humans, the IL-1 gene is located on chromosome 2 and its encoded protein consists of two bioactive ligands, IL-1α and IL-1β, with two cell surface receptors, the IL-1 type 1 (IL-1R1) and type 2 (IL-1R2) receptors and a natural receptor antagonist, IL-1RA (Webb, Collins et al. 1986). IL-1α and IL-1β share 26% and 45% homologies of amino acid and nucleotide sequence respectively and have comparable biological effect, playing an essential role in the inflammatory process and the immune response (Dower, Kronheim et al. 1986). IL-1RA is a secreted glycosylated protein of 22 kDa (Hannum, Wilcox et al. 1990), which can bind to IL-1Rs with the same affinity as IL-1 but without initiating downstream signalling (Arend and Guthridge 2000). The main function of IL-1RA is to regulate the effects of IL-1 by blocking receptor signalling. IL-1α, IL-1β and IL-1RA bind to membrane receptors localized on target cells. IL-1R1, an 80kDa glycoprotein and IL-1R2, a 60kDa protein belong to the immunoglobulin family. Although the homology of the two receptors is only 28%, they can both bind the two agonist and antagonist ligands with high affinity, because their extracellular domains share high similarity (McMahan, Slack et al. 1991). However, IL-1α, IL-1β and IL-1RA responses are exclusively mediated through IL-1R1, because IL-1R2 is a decoy receptor, unable to transduce signals due to the absence of a specific domain (Colotta, Re et al. 1993).

Several publications demonstrate that IL-1 can be produced in the ovary. The biological activity of IL-1 was measured for the first time in human follicular fluid by Khan (Khan, Schmidt et al. 1988). This result has been subsequently confirmed (Barak, Mordel et al. 1992; Wang and Norman 1992), and indicates local IL-1 production by granulosa and/or theca cells. Also, IL-1α is secreted by OSE (Ziltener, Maines-Bandiera et al. 1993). Moreover, cumulus cells were demonstrated to express IL-1α and IL-1β mRNAs in women undergoing IVF (de los Santos, Anderson et al. 1998).

IL-1 plays a major role throughout the ovarian cycle, in particular in the ovulatory process, during which it can regulate the synthesis of proteolytic enzymes, production of PGs and NO as well as steroidogenesis. As reviewed in the previous section, ovulation is a risk factor for ovarian cancer. Therefore, the pro-inflammatory effect of IL-1 in ovulation is proposed to be causal in the initiation and promotion of ovarian cancer. Since OSE is suggested to be the main origin of EOC, there have been many studies on the effect of IL-1 on OSE. IL-1α potentially promotes PG secretion and leads to follicular rupture that is associated with cell damage and genotoxicity by increasing cyclooxygenase (COX)-2 mRNA expression in OSE (Rae, Niven et al. 2004a). In addition, IL-1α up regulates IL-6 and IL-8 mRNAs; both encoded proteins being secreted as pro-inflammatory ovulation-associated factors. Also, lysyl oxidase (LOX) mRNA that is associated with tissue post-ovulatory repair as well as stimulation of nuclear factor-kappa B (NF-Kb) inflammatory pathway molecules is enhanced by IL-1α (Rae, Niven et al. 2004b). IL-1α depresses GnRH receptor mRNA expression (Rae, Niven et al. 2004b), through which GnRH regulates its anti-proliferative effects. Furthermore, IL-1α induces 11βHSD1 mRNA and enzyme activity, which converts inactive cortisone to cortisol, demonstrating a potential role of locally generated active glucocorticoid in the OSE to reduce tissue damage and promote post-ovulatory wound healing (Yong, Harlow et al. 2002; Rae, Niven et al. 2004a). By contrast, 11βHSD2 that is responsible for inactivation of cortisol by converting it to cortisone, is undetectable in primary OSE cells while its levels are significantly higher in ovarian cancer cell lines (Gubbay, Guo et al. 2004). Moreover, 11βHSD1 expression in cancer cell lines is not responsive to IL-1α. These

data imply that 11βHSD2 expression and lack of 11βHSD1 response to IL-1α stimulus might be features of ovarian neoplastic transformation (Gubbay, Guo et al. 2005).

There is evidence that IL-1α produced by EOC cells is involved in the development of EOC. The IL-1α level is elevated in the serum of patients with EOC (Zeisler, Tempfer et al. 1998; Kondera-Anasz, Mielczarek-Palacz et al. 2003) and IL-1α is detectable in ascites from ovarian cancer patients (Moradi, Carson et al. 1993). The levels of both IL-1α and IL-1β detected by immunohistochemical staining and their bioactivities are markedly higher in cancerous than in normal ovarian tissues. In addition IL-1α and IL-1β are secreted mainly by epithelial cancer cells (Huleihel, Maymon et al. 1997). Furthermore, IL-1α has been reported to stimulate growth of ovarian cancer cells in an autocrine manner (Marth, Zeimet et al. 1996; Kawakami, Nagai et al. 1997).

1.1.4.2 IL-4

IL-4 is a T lymphocyte-associated cytokine produced mainly by T helper 2 (Th-2) cells. It exerts its effect by binding to the IL-4 receptor (IL-4R), which belongs to the family of type I cytokine receptor and contains two polypeptide chains, the IL-4Rα chain and common γ chain (Nelms, Keegan et al. 1999). IL-4 participates in several cell immune responses with a central role in mediating the differentiation of antigen-stimulated naive T cells. IL-4 also plays an important role in tissue adhesion and inflammation as well as regulating inflammation-associated diseases such as cancer by decreasing Th-1 responses (Nelms, Keegan et al. 1999).

OSE cells seem unable to secrete IL-4 although IL-4R mRNA has been detected (Ziltener, Maines-Bandiera et al. 1993; Burke, Relf et al. 1996). IL-4 in the pre-menopausal ovary is mainly secreted by the peripheral blood mononuclear cells that infiltrate the peri-ovulatory follicle and later the corpus luteum. The IL-4 level appears to be progesterone responsive and may have a role in post-ovulatory ovarian repair during the luteal phase of the menstrual cycle and in the corpus luteum of pregnancy (Hashii, Fujiwara et al. 1998).

IL-4 has been shown to have a role in EOC. In one study, about 60% of 21 ovarian cancer tissue samples expressed high levels of IL-4R, while seven normal ovarian tissues tested express no or low levels of IL-4R. In addition, IL-4 cytotoxin prolongs survival of tumour-bearing mice, suggesting that IL-4R-targeted cytotoxin may be a potent agent for the treatment of EOC patients (Kioi, Takahashi et al. 2005). In another study in which a cytokine antibody array was used, the level of IL-4 was shown to be two times higher in the culture supernatants of EOC cell lines compared to those of OSE cells, indicating EOC can produce more IL-4 (Chen, Ye et al. 2009).

1.1.4.3 IL-6

IL-6, a pleiotropic inflammatory cytokine, is encoded by the IL6 gene located on chromosome 7. The action of IL-6 is mediated through binding to the IL-6 receptor (IL-6R), which is a type I cytokine receptor and consists of a heterodimeric complex made up of a IL-6R subunit and signal transducer, Glycoprotein 130. IL-6 regulates the differentiation of lymphocytes, cell proliferation, cell survival and apoptotic signals (Hodge, Hurt et al. 2005). In addition, it plays a role in general system metabolism, endocrine functions, and other tissues and organ systems (Hodge, Hurt et al. 2005). Depending on the cell type, IL-6 can act via several classic protein kinase cascades such as mitogen activated protein kinase (MAPK) and phosphatidyl-inositol-3 kinase (PI-3K) (Yang, Wang et al. 2003).

IL-6 is present in the normal ovary, where it may act in follicle development by reducing the FSH binding capacity of granulosa cells. It is suggested that intra-ovarian IL-6 may be secreted by lymphocytes (Machelon, Nome et al. 1994). There is evidence that IL-6 is secreted by OSE cells (Ziltener, Maines-Bandiera et al. 1993). IL-6 mRNA expression is also detected in OSE cells and this expression can be enhanced by IL-1α treatment (Rae, Niven et al. 2004b).

IL-6 is one of the most widely studied cytokines in ovarian cancer. There are increased levels of IL-6 in ascites of EOC patients in comparison to plasma (Giuntoli,

Webb et al. 2009). Serum IL-6 levels are significantly higher in ovarian cancer than in endometriosis or benign tumours (Darai, Detchev et al. 2003). Women with advanced cancer have remarkably higher serum IL-6 levels than women with early stages or benign tumours. The concentrations of IL-6 are higher in ascites of cancer patients than in ascites of women with benign tumours (Nowak, Glowacka et al.). Two proposed major cellular tumour-associated sites of IL-6 production are peritoneal mesothelial cells and cancer cells (Offner, Obrist et al. 1995). Also, a correlation was found between enhanced levels of IL-6 in the cystic fluids of ovarian cancer patients and inhibited haemoglobin levels, which is another poor prognostic factor for EOC (van der Zee, de Cuyper et al. 1995). However, IL-6 appears not to stimulate tumour growth directly, because anti-IL-6 antibody fails to affect tumour cell proliferation *in vitro* (Watson, Sensintaffar et al. 1990). In contrast, IL-6 is demonstrated to enhance the invasiveness of ovarian cancer cells significantly by increasing their attachment and migration (Obata, Tamakoshi et al. 1997).

1.1.4.4 IL-8

IL-8, also known as CXCL8, is a proinflammatory chemokine. Transcription of the IL- 8 gene encodes for either a 72 amino acid protein in monocytes and macrophages or a 77 amino acid form in nonimmune cells. Expression of IL-8 is principally mediated by activator protein and/or nuclear factor-κB-mediated transcriptional activity (Xie 2001). Additionally, IL-8 is regulated by other factors including inflammatory signals, chemotherapy agents and hypoxia, as well as steroid hormones such as androgens and oestrogens (Xie 2001). IL-8 is a ligand for two cell-surface G protein–coupled receptors, CXCR1 and CXCR2 (Holmes, Lee et al. 1991). As a member of the CXC chemokine family, IL-8 plays an important role as an activator and chemoattractant for neutrophils. IL-8 is mainly produced by monocytes/macrophages, but also by other cells. Its expression occurs in many human cancers, including breast, colon, cervix, lung, ovary, prostate and renal cell carcinoma (Xie 2001).

There is evidence that IL-8 plays an important role in ovulation. In one study high concentrations of IL-8 were detected in follicular fluid of women hyperstimulated

during gonadotrophin-induced follicle growth (Runesson, Bostrom et al. 1996) and also in ovulatory follicles collected from the natural human menstrual cycle (Runesson, Ivarsson et al. 2000). Moreover, IL-8 is secreted from theca and granulosa cells isolated from preovulatory follicles, with significantly higher secretion from the theca cells (Runesson, Ivarsson et al. 2000). Finally, IL-8 mRNA expression is detected in OSE and its expression is up-regulated by IL-1α treatment (Rae, Niven et al. 2004b).

There are also several lines of evidence to suggest the involvement of IL-8 in the progression of EOC. Firstly, enhanced levels of IL-8 are detected in ascites of ovarian cancer patients compared to plasma (Giuntoli, Webb et al. 2009). Secondly, the levels of IL-8 are markedly stimulated in cyst fluid from malignant in comparison to benign tumours (Nowak, Glowacka et al.; Ivarsson, Runesson et al. 1998). Thirdly, most EOC cell lines constitutively express IL-8 (Toutirais, Chartier et al. 2003). Lastly, expression of IL-8 protein in the tissue increases with lower differentiation status of the tumours. Both types of IL-8 receptors are detected in most specimens. There is a typical expression pattern for IL-8 receptor A, with expression limited to the luminal side of the epithelial tumour cells, whereas IL-8 receptor B is more equally distributed in the samples (Ivarsson, Ekerydh et al. 2000). Nearly half of 102 cancer samples have high IL-8 expression and the remainders have little or no IL-8 expression. High IL-8 expression is associated with advanced tumour stage, high tumour grade and poorer survival. Ovarian cancer growth can be inhibited through antiangiogenic mechanisms by silencing IL-8 gene expression (Merritt, Lin et al. 2008).

1.1.4.5 IL-10

The human IL-10 gene is located on chromosome 1 and encodes for 5 exons (Spits and de Waal Malefyt 1992). IL-10 exerts its action by binding to its specific cell surface receptor, which is composed of two subunits, α and β, that are members of the interferon receptor (IFNR) family (Kotenko, Krause et al. 1997). The receptor is present on many types of cell, in particular immune cells. The IL-10 promoter contains several transcription factor-responsive elements. Thus the production of IL-

10 by the cells can be promoted by several endogenous and exogenous factors such as endotoxin, TNF-α, catecholamines, and 3'-5'-cyclic adenosine monophosphate (cAMP)-elevating drugs (Asadullah, Sterry et al. 2003). Particularly, the stress axis is an important regulator of IL-10 expression *in vivo*. The main biological effect of IL-10 is to limit and terminate inflammatory responses and then regulate the differentiation and proliferation of immune cells such as T cells, B cells, natural killer cells and granulocytes (Asadullah, Sterry et al. 2003). In addition, many investigations indicate a major role of IL-10 in inflammatory and malignant diseases. Enhanced expression of IL-10 is found in many tumours and is considered to promote further tumour development.

There have been several reports of increased IL-10 expression in ovarian cancer. In one study, enhanced levels of IL-10 were detected in ascites of ovarian cancer patients compared to plasma (Giuntoli, Webb et al. 2009). In another study, ten of eleven tumour biopsies expressed mRNA of IL-10 whereas the eight normal ovaries studied did not (Pisa, Halapi et al. 1992). In other studies, total mRNA expression of IL-10 was detected in 17 of 20 malignant ovarian cancer specimens while there was no expression in 10 benign samples (Merogi, Marrogi et al. 1997). It was also reported that a majority of solid EOC samples expressed transcripts for IL-10 (Nash, Lenzi et al. 1998).

1.2 Ovarian cancer

1.2.1 Clinical features of ovarian cancer

1.2.1.1 Incidence and mortality

Ovarian cancer is the 5th most common cancer in women, after breast, bowel, lung and uterine cancer, with approximately 6720 women being diagnosed in 2007 in the UK (Cancer Research UK Statistics). It has the highest mortality rate of all gynaecological cancers and is the 4th most common cause of cancer related death in women in the UK. About 4,370 women died of ovarian cancer in 2008 in the UK (Cancer Research UK Statistics). The main reason for this poor prognosis is the rapid

and asymptomatic progression of the tumour along with the absence of efficient screening tests, resulting in its presence at late stage of disease and less responsive to treatment and cure. Ovarian cancer can occur at any age but is predominantly a disease of older, post-menopausal women with over 80% of cases being diagnosed in women over 50 years, peaking in women aged 65 and over (Cancer Research UK Statistics).

1.2.1.2 Classification of ovarian cancer

According to the World's Health Organization (WHO) Classification of Ovarian Tumours, made in 1973 and updated in 1999, ovarian tumours can be classified into three major categories: surface epithelial-stromal tumours, sex cord-stromal tumours and germ cell tumours, depending on their originating anatomic structure. Tumours with combinations of different subtypes either intermixed or side-by-side within a single tumour can sometimes be found and are designated as mixed, with the contributing subtypes specified in the designation. Among various ovarian tumours, the most common types are surface epithelial-stromal tumours, also known as epithelial ovarian tumours, which originate from the surface epithelium of the ovary and account for approximately 60% of all the ovarian tumours and 90% of malignant ovarian tumours. Depending on histological features, epithelial ovarian tumours are subcategorised into five major subtypes, serous, mucinous, endometrioid, clear cell and transitional cell tumours. Highly malignant epithelial-stromal tumours lacking any specific differentiation are classified as undifferentiated. Each subtype of epithelial ovarian tumour can be further classified as benign if they lack exuberant cellular proliferation and invasive behaviour; borderline if there is exuberant cellular proliferation but no invasive behaviour and malignant if there is invasive behaviour. The malignant epithelial ovarian tumours, also named epithelial ovarian cancer or carcinoma (EOC), will be introduced in detail as follows.

The most common types of EOC are malignant serous tumours, representing approximately 50% of all malignant ovarian tumours. Serous ovarian carcinomas are classified as tumours that resemble the epithelium of the oviduct or Fallopian tube.

Most of them are partially cystic, containing multiple cyst chambers and also solid areas, and display an abundance of delicate papillae that project into the cyst cavities or outward from the external surface of the tumour. Most of them occur in women in their sixties and two-thirds of them are bilateral.

Malignant endometrioid ovarian tumours, which have features of endometrial glandular epithelium, are the second most common EOC, accounting for 10–25% of EOC. They may be cystic or predominantly solid. Most of the tumours are confined to the ovaries and adjacent pelvic structures and are considered to have a better prognosis than either mucinous or serous carcinomas. These tumours usually appear in the sixth decade of women's life and about 13–28% of them are bilateral.

Malignant mucinous tumours, which have features of the uterine cervical or intestinal epithelium, represent 5–10% of EOC. Malignant mucinous tumours may contain papillary projections within the cyst cavities, larger solid areas, and necrosis and haemorrhage regions as well. Most of them appear in the sixth decade of life and 6%-20% of malignant mucinous tumours are bilateral. Late extra-peritoneal recurrences, particularly in the lungs, are characteristic of malignant mucinous tumours.

Malignant clear cell tumours consisting of cells with clear cytoplasm make up 4–5% of EOC. Usually, they are diagnosed in the fifth decade of life. Two-thirds of all women with this type of tumours are nulliparous, and 50–70% of them are affected by endometriosis. Survival rates for clear cell carcinomas are poorer than the other EOC.

Transitional cell tumours consist of cells that resemble transitional epithelium or urothelium of the urinary bladder. They contain solid and cystic regions with internal papillary projections. About one-tenth of these tumours are bilateral. They are rare and likely to have excellent prognosis.

1.2.1.3 Stage and grade of ovarian cancer

Staging of ovarian tumour, also known as the extent of tumoural spread, is performed according to the TNM system, with T standing for extent of primary tumour, N standing for degree of spread to lymph nodes and M standing for metastasis (Flemming ID 1997). The system is established by the American Joint Committee on Cancer (AJCC) and it is comparable to the staging system approved by the International Federation of Gynaecology and Obstetrics (FIGO) (Table 1.1). This international system of staging of the ovarian cancer is useful for therapeutic strategies, prognosis of the disease and also clinical research, which need comparable global standard.

The grade (G) of ovarian cancer, also known as the degree of tumour differentiation, represents the degree to which the tumour has similarity to the normal tissue. G1 refers to tumours that are well-differentiated, demonstrating little cellular atypia and few mitoses. G2 refers to moderately-differentiated tumours, exhibiting enhanced cellular atypia and more frequent mitoses but retention of histological differentiation. G3 refers to poorly-differentiated tumours with frequent mitoses, and containing poorly or undifferentiated cells with few distinguishing features (Chen, Ruiz et al. 2003). The grading of the ovarian tumour is useful for prognosis and therapeutic strategies.

AJCC	FIGO	Description
TX		Primary tumor cannot be assessed.
T0		No evidence of primary tumor.
T1	I	Tumor limited to ovaries (one or both).
T1a	IA	Tumor limited to one ovary; capsule intact, no tumor on ovarian surface. No malignant cells in ascites[c] or peritoneal washings.
T1b	IB	Tumor limited to both ovaries; capsules intact, no tumor on ovarian surface. No malignant cells in ascites or peritoneal washings.
T1c	IC	Tumor limited to one or both ovaries, with any of the following: capsule ruptured, tumor on ovarian surface, malignant cells in ascites or peritoneal washings.
T2	II	Tumor involves one or both ovaries with pelvic extension.
T2a	IIA	Extension and/or implants on uterus and/or tube(s). No malignant cells in ascites or peritoneal washings.
T2b	IIB	Extension to other pelvic tissues. No malignant cells in ascites or peritoneal washings.
T2c	IIC	Pelvic extension (2a/IIA or 2b/IIB) with malignant cells in ascites or peritoneal washings.
T3 and/or N1	III	Tumor involves one or both ovaries, with microscopically confirmed peritoneal metastasis outside the pelvis and/or regional lymph node metastasis.[d]
T3a	IIIA	Microscopic peritoneal metastasis beyond pelvis.
T3b	IIIB	Macroscopic peritoneal metastasis (2 cm or less in greatest dimension) beyond pelvis.
T3c and/or N1	IIIC	Peritoneal metastasis (more than 2 cm in greatest dimension) beyond pelvis and/or regional lymph node metastasis.
M1	IV	Distant metastasis (excludes peritoneal metastasis).[e]

Table 1.1: Staging of ovarian cancer according to AJCC and FIGO. Adapted from Chen *et al.* (2003)

1.2.1.4 Treatment

The standard care for EOC patients involves staging and cytoreduction surgery followed by chemotherapy with a platinum and taxane combination (Williams, Toups et al. 2007). Adjuvant treatments such as radiotherapy and hormonal therapies have demonstrated effectiveness in certain patients.

The main aims of surgery for primary cancer are accurate diagnosis, staging and removal of tumour to the maximum extent. The surgical plans are variable depending on the individual stages and histology subtypes. In the treatment of advanced disease, cytoreductive surgery, which eradicates tumour incompletely but can improve the chances of response to chemotherapy, is a common intervention. The main procedures include, but are not limited to, total abdominal hysterectomy (removal of the uterus), bilateral salpingo oophorectomy (removal of fallopian tubes and ovaries), infracolic, omentectomy (excision of the omentum beneath the transverse colon), and

lymph-adenectomy (lymph node dissection) (Stratton, Tidy et al. 2001). The overall survival of EOC patients can be significantly increased if cytoreductive surgery decreases tumour size to less than 1 cm, and the size of any remaining tumour is a very important prognostic factor (Cannistra 1993).

Standard chemotherapy usually consists of a combination of Carboplatin and Paclitaxel. Carboplatin elicits cell death by forming inter- and intra-strand cross-links with DNA or DNA adducts and also by disrupting DNA replication. Furthermore, Carboplatin activates signal transduction pathways by damaging the protein structure of receptor or ligand molecules by interaction with their thiol groups. This drug can also damage DNA beyond repair, causing apoptosis (Williams, Toups et al. 2007). Paclitaxel acts through hyperstabilization of microtubules, thus compromising flexibility of the cellular cytoskeleton (Williams, Toups et al. 2007). Chemotherapy frequently produces clinical remissions but relapse is a common event and when drug resistance occurs it is usually fatal (Kaye 1996). Studies have shown that approximately 50-75% of patients will eventually experience a relapse of EOC (Johnston 2004). Thorough understanding of underlying mechanisms by which the drug resistance develops, and development of therapeutic drugs for resistant EOC, remain active areas of research.

Radiotherapy and hormonal therapies have demonstrated benefit for some patients. Radiotherapy is usually used as a palliative treatment for the recurrent ovarian cancer patients, controlling symptoms such as bleeding and pain. The main reason for the limited use of radiotherapy in EOC is that spread of the disease throughout the peritoneal cavity limits the delivery of safe doses.

Hormonal therapies have often been applied as a safe and effective complementary or alternative treatment for recurrent ovarian cancer. Tamoxifen, a synthetic selective ER modulator, which acts by competitive binding of ER and used widely in the treatment of breast cancer, has also been used in treatment of ovarian cancer. A review of 18 studies including 648 patients found the overall response rate to tamoxifen was 13%, including a 4% complete response rate, a 9% partial response

rate, and 38% stabilization of disease (Perez-Gracia and Carrasco 2002). Tamoxifen usually has a mild toxicity so it can be a choice for heavily treated patients with recurrent disease, especially for those with poor response. Aromatase inhibitors (AIs) like letrozole and anastrozole have been tested in phase II clinical trials in which AIs have been conducted in recurrent or persistent ovarian cancer (Bowman, Gabra et al. 2002; del Carmen, Fuller et al. 2003; Papadimitriou, Markaki et al. 2004). The results indicate that AIs have modest activity in recurrent ovarian cancer. Similar to other hormonal therapies, progesterone has been tried mostly in recurrent ovarian cancer patients who have failed one or more chemotherapeutic therapies. A review of 13 studies including 432 patients treated by megestrol acetate (MA) or medroxyprogesterone acetate (MPA) revealed a complete response rate of 2.3%, a partial response rate of 4.9% and stable disease in 10.9% of patients (Zheng, Kavanagh et al. 2007). High-dose progesterone therapy (MA, 400–800 mg/day) has not been proven to be more effective than regular dose (MA, 160 mg/day) (Veenhof, van der Burg et al. 1994). RU486 (mifepristone) is a synthetic antiprogestin which competitively binds the PR. It has been tested in one study in the treatment of cisplatin and paclitaxel-resistant ovarian cancer patients. There is an overall response rate of 26.5%, including a complete response rate of 9% and partial response in 17.5% of patients. In addition, a few clinical trials have shown GnRH agonists (leuprolide acetate, goserelin, triptorelin) may provide modest efficacy in patients with relapsed ovarian cancer. As treatment with a GnRH agonist has few side effects, it can be a valid choice in patients who cannot tolerate or accept chemotherapy (Emons, Grundker et al. 2003; Grundker and Emons 2003). A clinical trial of the GnRH antagonist cetrorelix has shown it has a clear effect on refractory ovarian cancer and may be tried on more patients in the future (Verschraegen, Westphalen et al. 2003). Lastly, the effectiveness of antiandrogen therapy has been explored in a small number of studies. Response rate and disease stabilization of ovarian cancer patients to flutamide (nonsteroidal antiandrogenic drug) are 4.3% and 8.7% in one study (Vassilomanolakis, Koumakis et al. 1997), and 6.3% and 28% in another study (Tumolo, Rao et al. 1994), indicating some effect in relapsed ovarian cancer.

1.2.2 Origin of ovarian cancer

Traditionally, OSE and OSE lining inclusion cysts are thought to be the source of EOC (Auersperg, Wong et al. 2001; Murdoch and McDonnel 2002). The evidence for EOC arising from OSE is indirect, based on the epidemiological finding that the risk of developing EOC rises with increased number of lifetime ovulations and OSE is repetitively injured during each ovulation (Espey 1994; Ness, Grisso et al. 2000; Fleming, Beaugie et al. 2006). Many authors hypothesize that physical trauma inflicted by ovulation and DNA damage caused by ovulation-associated inflammatory cytokines and reactive oxygen species increase OSE vulnerability to genetic mutation and susceptibility to oncogenic transformation. This is supported by experiments showing that OSE, from both rats and mice, which has been continuously cultured to mimic the repetitive injury and repair that OSE undergoes spontaneously transforms into cancerous cells (Godwin, Testa et al. 1992; Testa, Getts et al. 1994; Roby, Taylor et al. 2000).

After ovulation, epithelial inclusion cysts are formed by invaginations of OSE into the cortical stroma, where they become trapped. There is some evidence linking their presence to EOC development. Firstly, inclusion cysts are more numerous in patients with family histories of ovarian cancer (Mittal, Zeleniuch-Jacquotte et al. 1993; Werness, Afify et al. 1999). Secondly, the incidence of inclusion cysts is significantly higher in normal ovaries with contralateral ovarian tumours, compared with those examined after incidental oophorectomy, 82% vs. 61% (Okamura and Katabuchi 2001). Thirdly, inclusion cysts are more frequently observed in post-menopausal women than in premenopausal women, 66% vs. 51% (Okamura and Katabuchi 2001). Since the peak incidence of EOC is after menopause, the occurrence rate of inclusion cysts is intriguing. Moreover, mitotic activity is demonstrated in some OSE cells lining the inclusion cysts by immunohistochemical methods, and p53 protein is also present in such OSE cells (Okamura and Katabuchi 2001). Finally, c-myc gene is reportedly activated in the OSE cells of inclusion cysts in normal human ovaries (Tashiro H 1993). Consequently, in response to genetic alterations and the accumulation of additional mutant proteins, especially in inclusion cysts resulting from the process of surface epithelium repair as a consequence of

ovulation, the inclusion cysts may become committed to form preneoplastic lesions (Okamura, Katabuchi et al. 2006).

Inside the ovary, inclusion cysts are exposed to a hormone-rich environment, where gonadotrophins, androgen, progesterone and oestrogen act together and induce proliferation of OSE lining the inclusion cyst, eventually giving rise to ovarian carcinoma (Wong and Leung 2007). The effect of different sex steroids will be reviewed in detail in next section.

The OSE-inclusion cyst model can explain many important features of ovarian tumourigenesis, however, it also has its limitations. For instance, it does not explain why invasive endometrioid and mucinous carcinomas are frequently associated with borderline tumours in the ovary, while invasive serous carcinomas are not (Karst and Drapkin 2010). Moreover, this model does not address the clear differences in genetic alterations that exist between tumour subtypes. If all ovarian tumours derive from inclusion cysts, why do they have such different genotypes and outcomes (Karst and Drapkin 2010)?

The fimbrial end of the fallopian tubes has also recently been proposed as a possible origin of EOC (Brown and Palmer 2009; Karst and Drapkin 2010). This concept arises from the studies exploring occult ovarian and fallopian tube cancer in women with breast cancer (BRCA) gene mutations. Inherited mutations in BRCA1 or BRCA2 are associated with familial ovarian cancer, which accounts for 11–15% of ovarian carcinomas (Risch, McLaughlin et al. 2006) and mutations in either gene hold a 15–40% lifetime risk of developing ovarian cancer (Wooster and Weber 2003). These results indicate that many ovarian tumours may actually arise from the distal region of the fallopian tube, but afterwards spread to the adjacent ovary. However, by the time of surgery the origin of the primary tumour may be difficult to identify.

1.2.3 Hormones and ovarian cancer

Based on epidemiological data, three hypotheses exist to explain the initiation and development of EOC. In addition to the incessant ovulation and inflammation hypotheses discussed in previous sections, gonadotrophins and sex hormones are thought to play roles in the pathogenesis of EOC, as will now be considered.

1.2.3.1 Gonadotrophins

The gonadotrophins, FSH and LH, are glycoprotein hormones synthesized in the anterior pituitary that play key endocrine roles in regulating ovarian steroidogenesis and gametogenesis. They are also suggested regulators of EOC. The 'gonadotrophin hypothesis' is one of the three hypotheses that have been proposed to explain ovarian cancer pathogenesis. It proposes that ovarian cancer develops as a result of excessive stimulation of ovarian tissue by gonadotrophins. Gonadotrophins may directly activate gonadotrophin-responsive genes in those cells experiencing malignant transformation or indirectly stimulate sex steroids that could influence malignant transformation through paracrine or endocrine mechanisms (Risch 1998).

Epidemiologic studies indirectly support the gonadotrophin hypothesis. Decreased risk for ovarian cancer is associated with breastfeeding and oral contraceptive use, both of which are associated with suppressed secretion and reduced exposure to gonadotrophins ((Rao and Slotman 1991; Rossing, Daling et al. 1994; Shoham 1994). Conversely, increased risk of ovarian cancer is associated with conditions where gonadotrophin levels are elevated, such as in post-menopausal women, in women who have received treatment for the induction of ovulation and in women with polycystic ovarian syndrome (PCOS) (Whittemore, Harris et al. 1992; Balen 1995). However, the lack of increase in risk related to early age at menopause and with twin pregnancies, both of which are associated with increased gonadotrophin levels (Martin, Robertson et al. 1991; Lambalk, Boomsma et al. 1998), and decreased blood levels of FSH and LH in ovarian cancer cases compared with controls (Helzlsouer, Alberg et al. 1995), indicate that gonadotrophins may not be responsible for changes in ovarian cancer risk directly.

There is also experimental evidence suggesting that gonadotrophins might act directly on OSE and thereby contribute to EOC. For example, gonadotrophins have been shown to enhance tumour angiogenesis and adhesion in ovarian cancer cells (Schiffenbauer, Meir et al. 2002; Zygmunt, Herr et al. 2002). However, the direct effect of gonadotrophins on the proliferation of normal OSE cells and ovarian cancer cells is still controversial, with different studies showing either an increase (Syed, Ulinski et al. 2001; Choi, Kang et al. 2002) or no effect (Zheng, Lu et al. 2000; Ivarsson, Sundfeldt et al. 2001). Two complementary DNA (cDNA) microarray studies which investigated the gene expression profiles of normal and neoplastic cells following FSH treatment, have shown FSH regulates a set of oncogenic and tumour-suppressor genes, although no matching genes were found between the two studies (Ho, Lau et al. 2003; Ji, Liu et al. 2004).

In conclusion, more experimental evidence is needed to establish the role of gonadotrophins in normal OSE biology and ovarian cancer development, to understand their possible mechanisms of action.

1.2.3.2 Androgens

Androgens are also implicated in the pathogenesis of ovarian cancer. Various epidemiologic data indicate a cancer promoting effect of androgen. Women taking oral contraceptives, which decrease androgen levels, have a decreased risk of ovarian cancer (Coenen, Thomas et al. 1996). Conversely, women with PCOS, a disease associated with hyperandrogenaemia, have a higher risk of developing ovarian cancer (Schildkraut, Schwingl et al. 1996). Moreover, patients with endometriosis, treated with Danazol (which depresses secretion of gonadotrophins but has androgenic properties) have 3-fold elevated risk of ovarian cancer compared with patients with no treatment, while patients who take leuprolide/nafarelin (GnRH agonists) are not at significantly elevated risk in comparison to patients with no medication (Cottreau, Ness et al. 2003).

Experimental data also support the concept that androgens are involved in the development of ovarian cancer. Thus AR is expressed in the majority of ovarian cancers (Chadha, Rao et al. 1993; Cardillo, Petrangeli et al. 1998) and several *in vitro* studies have shown androgen can promote cell proliferation of normal OSE cells (Karlan, Jones et al. 1995; Syed, Ulinski et al. 2001; Edmondson, Monaghan et al. 2002). Moreover, 5α-dihydrotestosterone (5α-DHT) reverses the growth-inhibitory effect of transforming growth factor-β1 (TGF-β1) on SKOV-3 cells and primary ovarian cancer cells recovered from ascitic fluid, suggesting that androgens may help ovarian cancer cells to escape from the growth inhibitory effects of TGF-β1 (Evangelou, Jindal et al. 2000). Furthermore, in an *in vivo* study, the growth of ovarian epithelial cells of guinea pigs was stimulated by testosterone treatment, resulting in the production of benign cysts and small adenomas in the ovarian parenchyma and papillomas on the ovarian surface (Silva, Tornos et al. 1997).

In conclusion, preliminary epidemiologic and experimental data have indicated accelerating effects of androgen in ovarian cancer development but more studies are necessary to ascertain a definitive role.

1.2.3.3 Progesterone

Epidemiologic evidence suggests that progesterone (P4) might protect against ovarian tumour development. Multiparity, associated with high levels of P4 in every pregnancy, has been reported to reduce the risk of ovarian cancer (Adami, Hsieh et al. 1994; Salazar-Martinez, Lazcano-Ponce et al. 1999). Moreover, twin pregnancy is also reported to be one protective factor of ovarian cancer. P4 levels during multiple pregnancies are higher compared with singleton pregnancies (Johnson, Abbas et al. 1994; Thomas, Murphy et al. 1998), and mothers of dizygotic twins may also have higher follicular phase serum P4 than women with single pregnancies (Gilfillan, Robertson et al. 1996). Furthermore, progestin-only oral contraceptives, which exert their effect through thickening of the cervical mucus and reduction of the endometrial receptivity to implantation, are reported to have a protective effect against ovarian cancer (Kumle, Weiderpass et al. 2004). Additionally, one study has shown that combination oral contraceptive formulations with high progestin potency

provide greater protection than those with low-progestin potency (Schildkraut, Calingaert et al. 2002), and risk of ovarian cancer is higher in users of oestrogen-only HRT than in combined oestrogen-progestin HRT users (Lacey, Mink et al. 2002; Riman, Dickman et al. 2002).

Experimental evidence is also consistent with a protective role of P4 in ovarian cancer. Firstly, i*n vitro* studies have demonstrated that higher doses of P4 inhibit cell growth in rhesus monkey OSE (Wright et al. 2002). Secondly, in addition to the negative role in cell growth, P4 also inhibits invasion of ovarian cancer cells. For example, P4 significantly reduced invasion of SKOV-3 cells into Matrigel and secretion of uPA (McDonnel and Murdoch 2001), and an *in vivo* study using athymic mice inoculated with SKOV-3 cells has show that treatment with P4 decreases invasion into host tissues (McDonnel, Van Kirk et al. 2005). Thirdly, the expression of PRs is associated with better survival of ovarian cancer and is considered to be a favourable prognostic marker in ovarian cancer (Lee, Rosen et al. 2005). The expression of PR-A protein was significantly lower in malignant tissue than in normal ovarian tissues, suggesting down-regulation of PR-A is associated with the development of ovarian epithelial carcinoma (Akahira, Suzuki et al. 2002). Lastly, recent cDNA microarray studies have revealed that P4 induces putative antitumorigenic genes including ATF-3 (an apoptosis inducer), NM23-H2 (a motility suppressor), and caveolin-1 and DLC-1 (tumour suppressors) (Syed, Mukherjee et al. 2005).

In summary, accumulating evidence indicates that P4 may be protective against ovarian cancer but once again, definitive confirmation is awaited.

1.2.3.4 Oestrogens

Oestrogens are most likely candidates as hormonal tumour promoters. The relationship between oestrogen-containing hormone replacement therapy (HRT) and the risk of ovarian cancer is still controversial. Some studies show a promoting effect (Negri, Tzonou et al. 1999) whilst others suggest no effect (Coughlin, Giustozzi et al. 2000). However, more recent epidemiological studies on post-

menopausal women have indicated that oestrogen replacement therapy (ERT) increases ovarian cancer incidence and mortality (Rodriguez, Patel et al. 2001; Lacey, Brinton et al. 2006; Beral, Bull et al. 2007).

Paradoxically, although oral contraceptives also involve administration of exogenous oestrogens, they show a protective effect on ovarian cancer. This may in part be because they also contain progestins, which as discussed above may be anti-tumourgenic. Oral contraceptives also suppress ovulation, which as discussed above would also confer protection. Apart from the contribution to suppression of ovulation and protective effect of progestins, another reason may be reduction of endogenous 17β-oestradiol (E_2) production by oral contraceptives. The 24-hour total serum ethinyl oestradiol (or equivalent) level in women on oral contraceptives is comparable to the naturally low E_2 level of the early- to mid-follicular phase (Humpel, Tuber et al. 1990; Dibbelt, Knuppen et al. 1991), which in turn is lower than late follicular- or luteal-phase E_2 levels. Breastfeeding is also likely to be protective with fewer ovulations, higher concentrations of progesterone and reduced serum concentrations of E_2 potentially combining to contribute to the protective effect (Liu, Rebar et al. 1983).

Classical oestrogen signalling, via oestrogen binding to ER located in the nucleus, induces transcriptional activation of oestrogen-regulated genes in ovarian cancer. Some of these genes are key components of the complex carcinogenesis process that includes proliferation, migration and invasion (Hayashido, Lucas et al. 1998). Up-regulation of c-myc has been shown to mediate oestrogen-induced ovarian cancer cell growth (Chien, Wang et al. 1994). Ezrin (Song, Fadiel et al. 2005), fibulin-1 (Clinton, Rougeot et al. 1996; Bardin, Moll et al. 2005), cathepsin D (Galtier-Dereure, Capony et al. 1992) and kallikreins (Yousef and Diamandis 2002), which are involved in motility and invasion of EOC cells, are reported to be regulated by oestrogen. In addition, oestrogen may interact with cytokines and growth factors in ovarian cancer cells. For example, oestrogen increases IL-6 mRNA and protein expression, resulting in the activation of the signal transducers and activators of transcription protein (STAT)-3 signalling pathway (Syed, Ulinski et al. 2002). An

EGF receptor-targeted antibody decreases the stimulatory effects of oestrogen on growth of PEO-1 ovarian cancer cells (Simpson et al. 1998). Oestrogen enhances EGF- and insulin growth factor (IGF)-I-mediated ovarian cancer cell growth by modulation of properties of receptors for the two growth factors (Wimalasena, Meehan et al. 1993). Lastly, E_2 decreases insulin-like growth factor binding protein (IGFBP)-3 and increases IGFBP-5 mRNA levels in PEO-4 ovarian cancer cells (Krywicki, Figueroa et al. 1993).

In summary, the epidemiological data together with experimental evidence indicate an involvement of oestrogen in the carcinogenesis and progression of ovarian cancer. The molecular mechanisms of the promoting effect and blockade of the effect by novel therapeutics will be of particular interest.

1.3 Oestrogen biosynthesis and mechanism of action

To appreciate the potential relevance of oestrogen signalling to the development of EOC it is necessary to understand the biochemical pathways of oestrogen formation and metabolism, the basis of which will now be assessed.

1.3.1 Pathway of oestrogen formation and metabolism

Endogenous oestrogens are responsible for the development and maintenance of the female reproductive system. Circulating oestrogens exist in a dynamic equilibrium of metabolic interconversion, but E_2 is the primary intracellular oestrogen and is substantially more active than either oestrone (E_1) or oestriol (E_3) at the receptor level. Before menopause, the primary source of oestrogen in adult women is the ovarian follicle through conversion of cholesterol, while after menopause, most endogenous oestrogen is formed by peripheral tissues through conversion of androstenedione (A), secreted by the adrenal cortex, to E_1 (Fig. 1.2). A major proportion of the oestrogens circulating in the body are conjugated (glucuronidated or sulphated) at hepatic sites in order to allow for rapid urinary excretion. Consequently, E_1 and oestrone sulphate (E_1S), are the most abundant circulating oestrogens in postmenopausal women. Furthermore, E_2 is produced and metabolized

locally via aromatase or STS pathways in some cancers and plays an important role locally in the development of these cancers. The underlying metabolic pathways in pre- and post-menopausal women will now be reviewed.

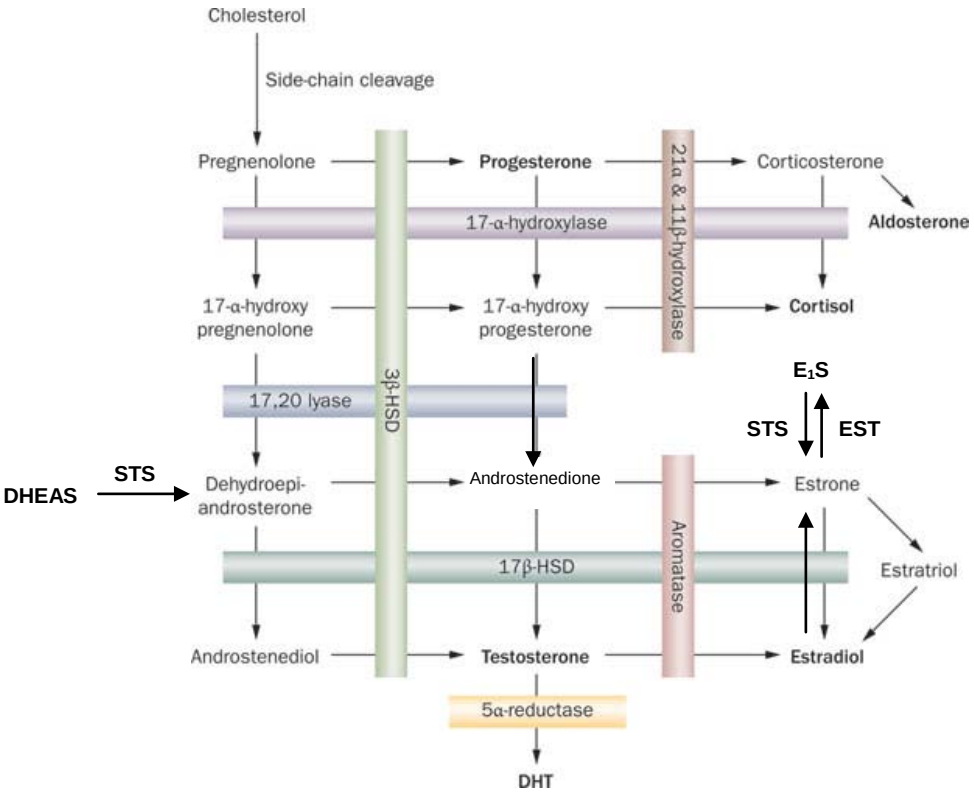

Figure 1.2: Major pathway of oestrogen biosynthesis and metabolism. Adapted from http://www.nature.com/nrurol/journal/v8/n7/fig_tab/nrurol.2011.67_F1.html#figure-title.
DHEAS: dehydroepiandrosterone sulphate; DHT: dihydrotestosterone; EST: oestrogen sulfotransferase; E_1S: oestrone sulphate; STS: steroid sulphatase; 3β-HSD: 3β hydroxysteroid dehydrogenase; 17β-HSD: 17β hydroxysteroid dehydrogenase.

1.3.1.1 Pre-menopausal oestrogen dynamics

In pre-menopausal adult women, most ostrogen is secreted by granulosa cells of the pre-ovulatory ovarian follicle and their derivatives (granulosa lutein cells) in the corpus luteum (Fig. 1.3). Small amounts of oestrogen are also produced by the adrenal cortex. During pregnancy, the placenta becomes the major source of oestrogen, necessary to sustain optimal feto-maternal development throughout gestation.

The cellular and biochemical interactions underpinning ovarian oestrogen biosynthesis and metabolism are summarized in Fig. 1.3.

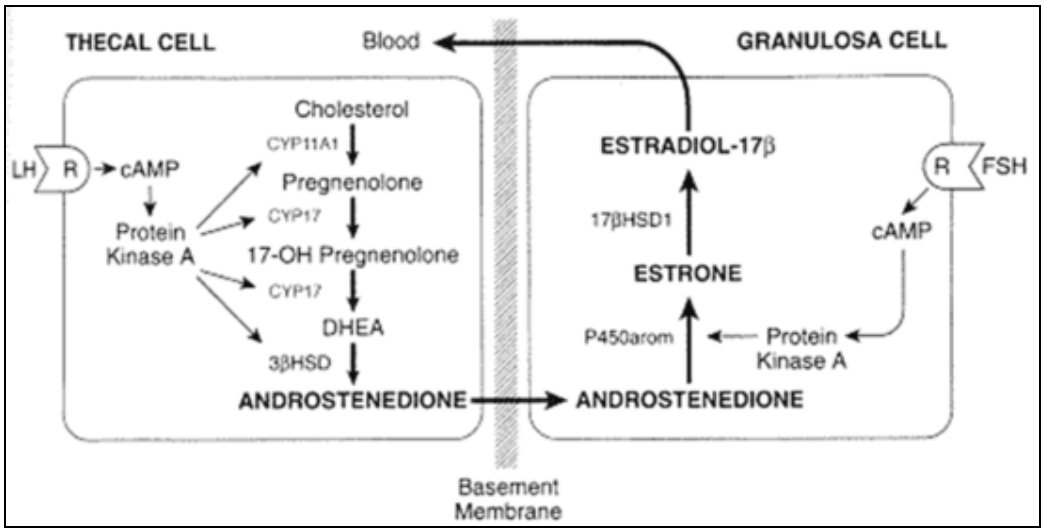

Figure 1.3: Two-cell, two-gonadotropin hypothesis of regulation of oestrogen synthesis in the human ovary. Adapted from http://www.endotext.org/female/female3/femaleframe3.htm. cAMP: 3'-5'-cyclic adenosine monophosphate; CYP11A1: cytochrome P450, family 11, subfamily A, polypeptide 1; CYP17: cytochrome P450, family 17, subfamily A, polypeptide 1; DHEA: dehydroepiandrosterone; FSH: follicle-stimulating hormone; LH: luteinising hormone; P450arom: aromatase; R: receptor; 3βHSD: 3β hydroxysteroid dehydrogenase; 17βHSD: 17β hydroxysteroid dehydrogenase type 1.

There are two peaks of oestrogen secretion during menstrual cycle. One is on day 12 or 13, before ovulation and the other is during the mid-luteal phase. The main effects of oestrogen are to promote growth of ovarian follicles, increase motility of uterine tubes, stimulate the growth of endometrium, enhance uterine blood flow, produce duct growth in the breasts and regulate the development of female secondary sex characteristics during puberty.

1.3.1.2 Post-menopausal oestrogen dynamics

In post-menopausal women, the ovaries cease to produce oestrogen, hence the ovary is no longer the main source of oestrogen. However, oestrogens are still produced at

a number of extragonadal peripheral sites, including fat, muscle, skin and liver, and by certain cancers of breast and endometrium (Miller 2004).

These sites express the aromatase enzyme necessary for conversion of androgens to oestrogens (Fig. 1.2), utilising precursor androgen produced by the adrenal cortex. Moreover, the post-menopausal ovary still produces substantial amounts of androgen, which can also be used as the precursor. Although aromatase activity has been identified in all of these tissues, levels remain substantially lower compared to primary endocrine organs like ovary and placenta. However, there are large masses of fat and muscle throughout the body and the combined metabolic capacity at these sites leads to the formation of micrograms of oestrogen per day. *In vivo*，muscle accounts for up to 30% and adipose tissue up to 15% of peripheral conversion of androgen to oestrogens (Miller 2004). Therefore, there is a positive correlation between body weight and serum oestrogen levels in post-menopausal women. In the same tissue of variable body areas, aromatase activities are different. For example, aromatase activity is much higher in adipose tissue from the buttocks than from the thighs, perhaps due to different local controls (Killinger, Perel et al. 1987).

The total quantity of oestrogen produced by these extragonadal sites may be relatively small but the local concentrations in the tissue are sufficient to act locally. As a consequence, circulating levels of oestrogens in post-menopausal women do not reflect direct oestrogen action in post-menopausal women.

1.3.1.3 Local production and metabolism of oestrogen

Various oestrogen-dependent cancers of the female reproductive tract have the ability to produce biologically active oestrogens. These locally formed oestrogens are considered to play a vital role in the development of such cancers by acting in the cells where they are produced without necessarily entering the extracellular space. This mechanism of formation and action, which differs from autocrine or paracrine action, is termed intracrine activation.

From many studies of breast cancers, we know there are two main pathways for the production of oestrogen *in situ*, the aromatase and STS pathways, which may also be involved in ovarian cancer. The start of the aromatase pathway would involve circulating dehydroepiandrosterone (DHEA) or A entering the cancer tissue. DHEA can then be converted to A by 3βHSDs or converted to androstenediol by 17βHSDs. A which comes from the circulation or converted from DHEA is then converted either to E_1 by aromatase or to testosterone (T) by 17βHSDs depending on the tissue. T is catalyzed further to E_2 by aromatase and E_1 is catalyzed to E_2 by 17βHSD1 or 17βHSD5 (Dufort, Rheault et al. 1999; Penning, Burczynski et al. 2000; Byrns and Penning 2009) depending on the tissue. In this pathway aromatase catalyzes conversion of A and T into E_1 and E_2 respectively. For the STS pathway, there are two sources of substrates. One substrate is E_1S which diffuses from the vessels. STS hydrolyzes E_1S to E_1. E_1 can subsequently be converted to E_2 by 17βHSD1 or 17βHSD5. Another substrate of STS is DHEAS which also diffuse from the vessels into the tissues. STS converts DHEAS to DHEA and DHEA is finally converted to E_2 via aromatase pathway. The resulting E_2 exerts its action on cancer cells through ER. At the site of active oestrogen synthesis, there may be opposing enzymes that convert active to inactive oestrogens. For example, oestrogen sulfotransferase (EST) which acts in the opposite direction to STS is responsible for the conversion of E_1 to E_1S or E_2 to E_2S. 17βHSD2 has the opposite effect to 17βHSD1 or 17βHSD5, converting E_2 to E_1 (Fig. 1.4). Therefore, the expression of oestrogen metabolizing enzymes producing biologically inactive oestrogen are equally important in determining the local oestrogen levels in cancers (Suzuki, Miki et al. 2005). In addition to the above enzymes, there is evidence that the uptake of E_1S by the cancer cells is mediated by special transporters, which are also important for the local formation of E_2. Studies of breast cancer and placenta have demonstrated OATP-B, OATP-D, OATP-E and OAT4 are the main candidate transporters of E_1S (Pizzagalli, Varga et al. 2003; Wlcek, Svoboda et al. 2008).

Both before and after menopause, there are high concentrations of circulating DHEAS, DHEA and A, which are precursor substrates of local oestrogen production via the aromatase pathway. DHEAS is secreted uniquely by the adrenal gland, with

circulating concentrations between 3μM and 12μM (Burger and Davis 2002). There are no significant changes in concentration during the menstrual cycle or related to the menopause (Burger et al. 2000). 50% of DHEA is formed by the adrenal gland, 20% by ovarian stroma and theca interna and the remaining is converted from circulating DHEAS by STS. The circulating level is in the range of 3-35nM (Burger and Davis, 2002). A is synthesized equally in the adrenal zona and ovary with the circulating levels between 2 and 8nM (Burger and Davis 2002). If there is STS and 3βHSD expression in the local area, DHEAS and DHEA which diffuse from the vessels can be converted to A, which therefore increases the concentration of A entering the local area directly from the vessels. The pre-menopausal E_1S serum concentration measured by radioimmunoassay is around 3nM in the follicular phase and 5nM in the luteal phase. After menopause it decreases to 0.4nM, but increases to 7nM in the women on HRT treatment (Ranadive, Mistry et al. 1998). Therefore the serum concentrations of precursors are high enough to facilitate the local production of significant amounts of oestrogen.

As discussed in Section 1.2.3.4, epidemiological data and experimental evidence suggest the involvement of oestrogen in the carcinogenesis and progression of ovarian cancer. Since the precursors for the aromatase and STS pathways exist, if the transporters and enzymes of these two pathways are present and active in ovarian cancer, E_2 can then be produced locally by cancer cells and consequently influence the development of ovarian cancer. Therefore it is very useful to investigate the expression and function of these enzymes and transporters involving in the two pathways to identify the specific important enzymes or transporter in ovarian cancer in order to apply relevant inhibitors to block the local formation of active estrogen to treat the disease effectively.

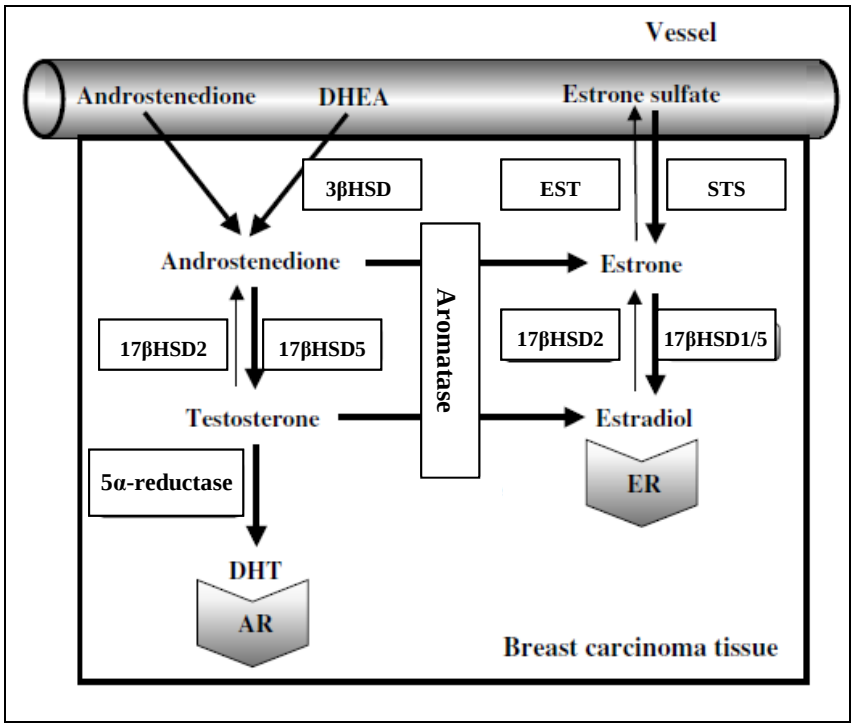

Figure 1.4: Representative pathways of *in situ* production of sex steroids in human breast carcinoma tissues. Adapted from Suzuki et al. (2005). AR: antrogen receptor; DHEA: dehydroepiandrosterone; DHT: dihydrotestosterone; ER: oestrogen receptor; EST: oestrogen sulfotransferase; STS: steroid sulphatase; 3βHSD: 3β hydroxysteroid dehydrogenase; 17βHSD: 17β hydroxysteroid dehydrogenase.

1.3.2 Mechanism of oestrogen action: receptors

Oestrogen exerts its major biological effect through interaction with classical ERs that reside mainly in the nucleus, although G protein-coupled receptor 30 (GPR30) has been proposed as a membrane-associated ER that mediates the nongenomic signalling of E_2. Since the studies of GPR30 are limited, only the classical ERs are reviewed in this section. There are two different forms of ERs, ERα and ERβ, encoded by different genes, ESR1 and ESR2 respectively. Classically, these receptor proteins function as transcription factors in the nucleus when they are bound to their respective ligands. ERα and ERβ have a similar structure that is shared by all the members of the steroid hormone receptor family. Both are composed of six functional domains A–F: the amino-terminal A/B domain with the hormone-

independent activation function (AF)-1; the middle C domain contains the DNA binding domain (DBD) which is responsible for ER binding to oestrogen response elements (EREs); the D domain, named the hinge region, is involved in co-regulatory protein binding; the carboxy-terminal domains E and F contain the ligand binding domain (LBD), which comprises the ligand-dependent transcription activation functions AF-2 (Sommer and Fuqua 2001).

Classically, the model for ER activation and inhibition is relatively simple. Upon agonist binding ER it undergoes conformational changes, and then the ER complex binds to DNA at the ERE site of specific genes which is highly specific for ER, and induces expression of the genes. Antagonists, on the other hand, compete for binding of agonists to the receptor and abrogate receptor interaction with target sequences, and this effect can be reversed by an excess of agonist (Sommer and Fuqua 2001). In the process, transcriptional activation of ER-regulated genes is mediated by the AF-1 and AF-2 regions, which can function either independently or synergistically in a promoter and target cell specific manner (Tora, White et al. 1989). Recently, an increasing number of co-regulatory factors that bind to ER have been discovered, facilitating or disrupting activation of target genes (McKenna, Lanz et al. 1999).

ERα and ERβ are co-expressed in many tissues, including the central nervous system, cardiovascular system, urogenital tract, breast, and bone. In the uterus and mammary gland, ERα is much more highly expressed than ERβ. In contrast, only ERβ is expressed in the gastro-intestinal tract (Gustafsson 1999). There have been many studies on ER expression in different cancers, especially in breast cancer. ERs are present in about 60-70% of breast tumours and are regarded as an important parameter in determining the hormonal sensitivity of the tumour and its likely response to anti-oestrogen therapy. The presence of ERs in ovarian cancers has also been studied. ERs are expressed in normal OSE cells and also in ovarian cancers (Hillier, Anderson et al. 1998; Pujol, Rey et al. 1998; Lau, Mok et al. 1999). Serous tumours are often ER-positive while mucinous and endometrioid tumours have lower amounts of ER (Quinn, Pearce et al. 1982). In addition, ERα is expressed in up to 60% of ovarian epithelial tumours and its levels are higher than in benign tumours or

normal ovaries (Willcocks, Toppila et al. 1983; Risch 1998). Moreover, expression of ERβ mRNA and protein has been reported in normal OSE cells as well as in ovarian cancer cell lines (Brandenberger, Tee et al. 1998). However, some studies suggest an increased ERα:ERβ ratio in ovarian cancer, which is thought to be a possible marker of ovarian carcinogenesis (Brandenberger, Tee et al. 1998; Pujol, Rey et al. 1998).

1.3.3 Therapeutic blockade of oestrogen action

It is well established that oestrogen has important roles in physiological and pathological conditions, especially an involvement in the risk towards and progression of oestrogen-related diseases like breast, endometrial and ovarian cancer. It is therefore not surprising that many studies have been carried out to develop potent inhibitors of oestrogen, which can be attractive measures both to prevent these diseases and to treat established tumours. Generally, there are two major strategies to influence the effect of oestrogen. One strategy is ER blockade by using drugs that block oestrogen signalling at the level of ER, thereby affecting oestrogen signal transduction. The other strategy is to reduce the biosynthesis of oestrogen. This approach can work by directly decreasing the conversion of androgen into oestrogen (inhibition of aromatase) or alternatively by blockade of the enzymes required for conversion of E_1S into E_1 (STS) or conversion of E_1 to E_2 (17βHSDs). In pre-menopausal women, reducing androgen substrate and aromatase in the ovary by LH releasing hormone agonist is an indirect method to reduce the production of oestrogen (Miller 2004) (Fig. 1.5). The following section will concentrate on the action of STS inhibitors.

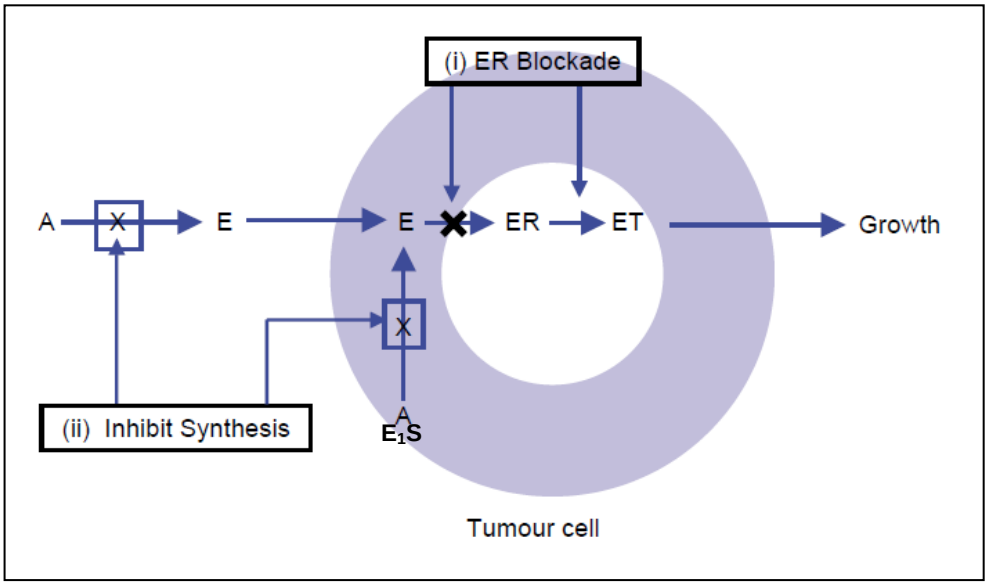

Figure 1. 5: Major strategies of reduction of oestrogen effect. (i) ER blockade (ii) inhibition of synthesis of oestrogen. Modified from Miller (2004). A: androgen; E: oestrogen; ER: oestrogen receptor; ET: oestrogen signal transduction; E_1S: oestrone sulphate.

Several potent irreversible STS inhibitors have now been identified, most of which have an aryl ring to which a sulfamate ester is attached as their active pharmacophore (Reed, Purohit et al. 2005). The first-generation STS inhibitor, STX64 (BN83495, also known as 667 Coumate, Fig. 1.6), is a nonsteroid-based tricyclic coumarin sulfamate. It is shown to block the stimulatory effect of E_1S on the growth of carcinogen-induced mammary tumours in ovariectomized rats (Purohit, Woo et al. 2000). Second-generation STS inhibitors, such as STX213 (Fig. 1.6), have now been developed. Its inhibitory effect on STS activity lasts for a much longer period of time than STX64 after a single dose in rodents (Fischer, Chander et al. 2003). These STS inhibitors are orally active with a high level of bioavailability, due to their hepatic transit without undergoing first-pass metabolism by binding to carbonic anhydrase II in erythrocytes after absorption (Ho, Purohit et al. 2003).

STX64 has been tested in one phase I clinical trial in post-menopausal women with advanced or metastatic breast cancer. The trial demonstrated that STX64 is well tolerated, with only a few minor side effects noted. At a dose of 5mg, more than 90% of STS activity was inhibited in blood lymphocytes, and the higher dose, 20mg, almost completely inhibited STS activity in blood lymphocytes and tumour tissue.

As expected, serum E_1, E_2 and androstenediol concentrations were significantly reduced with the administration of STX64. Unexpectedly, serum concentrations of A, the main aromatase substrate in post-menopausal women, were also reduced by up to 86%, suggesting that A in postmenopausal women derives mainly from peripheral conversion of DHEAS, not directly from the adrenal cortex (Stanway, Purohit et al. 2006). Since STX64 effectively blocks the production of androgens in addition to oestrogens in breast cancer, it is also likely to have therapeutic benefit in other hormone-dependent tumours, such as prostate, endometrial or ovarian cancer.

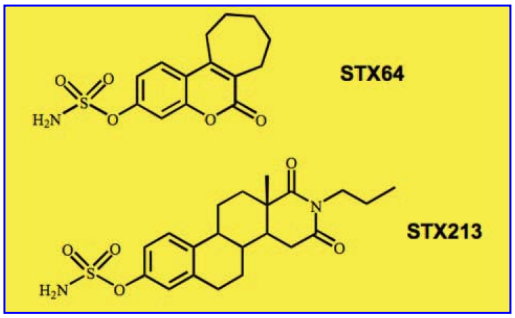

Figure 1.6: Molecular structures of STX64 and the second-generation steroid sulphatase inhibitor STX213. Adapted from Stanway et al. (2006)

1.3.4 Hormone replacement therapy

Women enter the period of menopause either by the natural cessation of ovulation or by artificial methods such as surgical intervention. The main changes include decreased ovarian oestrogen synthesis and an increase in gonadotrophin levels, resulting in some short-term symptoms including hot flushes and sweats, vaginal discomfort, recurrent lower urinary tract infection, urinary incontinence, and mood changes. Long-term consequences of menopause including cardiovascular disease, osteoporosis and Alzheimer's disease affect the health of post-menopausal women. HRT is medication prescribed to supplement the decreased levels of hormones in post-menopausal women to combat the symptoms. Primarily, HRT is used for short-term treatment of post-menopausal symptoms; as HRT become well studied, it has demonstrated protective benefits for some age-related disease, such as osteoporosis, heart disease, Alzheimer's disease, colon cancer and others, and therefore been prescribed for long-term treatment to protect the patients from these diseases.

However, apart from benefits, HRT has potential risks for the patients. It is reported HRT increase the risks of endometrial cancer (Grady, Gebretsadik et al. 1995), breast cancer (Beral, Banks et al. 1997) and venous thromboembolism (Perez Gutthann, Garcia Rodriguez et al. 1997). The relationship of HRT and ovarian cancer has been reviewed in Section 1.2.3.4. There are a few unwanted side-effects of HRT including weight gain, headaches, breast tenderness, vaginal bleeding, resulting in unwillingness to use HRT continuously.

HRT includes a variety of different regimens. In its simplest form, oestrogen replacement therapy (ERT) is used without combination of other hormones. Since oestrogens themselves are effective at relieving menopausal symptoms, women without a uterus need only take continuous oestrogens. However, for all women who have not had a hysterectomy, a progestogen is added for at least 10 days of each month to prevent endometrial hyperplasia and carcinoma. The sequential preparation, which is suitable for women in the peri-menopausal situation, has oestrogen in the first half of a 28-day cycle with progestogen in the second half. Continuous combined preparation, which is appropriate for those women who experience menopause for several years and wish to prevent vaginal bleeding, has oestrogen and progestogen daily. Other regimes prescribed to older women to protect their skeletal and cardiovascular system including tibolone, which combines oestrogenic and pregestogenic activity with weak androgenic actions and selective oestrogen receptor modulators (SERMs).

HRT is available in different preparations, such as tablets, patches, implants, gels, creams, and vaginal rings. Oral oestrogens are well tolerated, cheaper and are the most popular form. They are absorbed through the gut and transported to the hepatic portal circulation where most are metabolized by first pass effect. The disadvantages of oral oestrogens include nausea and variable levels due to hepatic metabolism, elevated serum triglyceride and a reduction in glucose tolerance. Oestrogens can be well absorbed through skin, subcutaneous fat and vaginal epithelium and these routes

of administration avoid the metabolism in liver, although they also have their own disadvantages, resulting in less use than oral forms.

There are three main oestrogens typically used for HRT: E_2, E_3 and conjugated equine oestrogens (CEEs). E_2 can be given by mouth, as a patch, implant or gel. CEEs and E_3 can only be administered by mouth (Studd John and Roger 1998). Preparations containing natural oestrogens are the most commonly used in Europe, while CEEs have been used most widely around the world, especially in North America (Sturdee 1997).

Due to their widespread use, the next section will concentrate on CEEs.

1.3.4.1 Conjugated equine oestrogens

CEEs contain a mixture of conjugated oestrogens obtained from urine of pregnant mares. Premarin is the commercial name of CEEs used for oral administration, in which the main ingredients are sodium oestrone sulphate (NaE_1S) and sodium equilin sulphate (NaEqS), comprising 48% and 26% respectively (Whittaker, Morgan et al. 1980). The other oestrogenic components are sulphated form of E_2, 17α-oestradiol, 17β-dihydroequilin, 17α-dihydroequilin, equilenin, 17β-dihydroequilenin, 17α-dihydroequilenin and delta 8-Oestrone (Bhavnani 2003). In addition, there are some carrier ingredients including calcium phosphate tribasic, calcium sulphate, cellulose, glyceryl monooleate, lactose, magnesium stearate and methylcellulose. Tablets are available in 0.3mg, 0.45mg, 0.625mg, 0.9mg, and 1.25mg. CEEs are well absorbed from the gastrointestinal tract after release from the tablet and are distributed and metabolized in the same manner as endogenous oestrogens. In one study of women taking Premarin, serum unconjugated oestrogens were measured by radioimmunoassay. Mean concentrations were E_2 393pM, E_1 599pM and equilin 6840pM (Whittaker, Morgan et al. 1980). The indications for usage include vasomotor symptoms, vulvar and vaginal atrophy associated with the menopause, hypoestrogenism due to hypogonadism, castration or primary ovarian failure and prevention of osteoporosis. The contraindications include undiagnosed abnormal vaginal bleeding, diagnosed, suspected, or history of breast cancer, oestrogen-

dependent neoplasia and pregnancy, liver diseases, and thromboembolic diseases (Introduction to Premarin, Wyeth Pharmaceuticals Inc.).

Given the prevalence of EOC in post-menopausal women, and the widespread use of CEE's discussed above, some of the known oestrogen responsive genes in cancer will now be discussed.

1.4 Oestrogen responsive genes in cancer

Oestrogen binding to its receptors activates post-receptor pathways, regulating the transcription of a number of genes involved in diverse aspects of cellular function including DNA repair, extracellular matrix remodelling, apoptosis and signal transduction. Identifying the downstream genes regulated by oestrogen will reveal the genetic basis of the wide range of biological activities of oestrogen, and provide a deeper understanding of the role of oestrogen in ovarian cancer. To date a few oestrogen-regulated genes have been revealed in ovarian cancer and the following sections will concentrate on reviews of four proteins encoded by oestrogen-regulated genes: insulin-like growth factor binding protein 3 (IGFBP3), Fibronectin (FN1), lysyl oxidase (LOX) and E-cadherin.

1.4.1 Insulin-like growth factor binding protein 3

IGFBP-3 is a component of the IGF system, comprising two growth factors, IGF-I and IGF-II; three cell membrane receptors, IGF-I receptor (IGF-IR), insulin receptor (IR), and IGF-II receptor (IGF-IIR); and six high-affinity binding proteins (IGFBP-1 to IGFBP-6) (Samani, Yakar et al. 2007). IGFBP-3 is predominantly expressed in the liver as well as a variety of non-hepatic normal and malignant tissues. Serum IGFBP-3 levels have been reported to be lower in patients with breast cancer (Bruning, Van Doorn et al. 1995) and colon cancer risk has been correlated with decreased IGFBP-3 levels (Ma, Pollak et al. 1999). Moreover, a prospective study showed that decreased serum IGFBP-3 levels are associated with the presence of high-risk colorectal adenomas, suggesting that the IGFBP3 may predict adenoma progression (Renehan, Painter et al. 2001). Furthermore, it has been suggested that

IGFBP-3 levels are reportedly lower in the serum of patients with ovarian cancer (Flyvbjerg, Mogensen et al. 1997).

In many cancer cell lines, IGFBP-3 has been demonstrated to inhibit IGF function. For example, IGFBP-3 activates a phosphotyrosine phosphatase that dephosphorylates IGF-IR, thereby decreasing its function in MCF-7 breast cancer cells (Ricort and Binoux 2002). Moreover, IGFBP-3 exerts an inhibitory effect on IGF-IR signalling by interfering with phosphatidyl-inositol-3 kinase (PI-3K)/Akt and MAPK pathways, resulting in induced apoptosis and arrested growth in non-small cell lung cancer cell lines (Lee, Chun et al. 2002). Furthermore, IGFBP-3 can also affect cell function in an IGF-independent manner. Thus, IGFBP-3 exerts IGF-I-independent growth-inhibitory effects in breast (Oh, Muller et al. 1993) and colon (Williams, Collard et al. 2000) cancers. Decreased IGFBP-3 levels can, therefore, influence cell growth both by reducing an inhibitory effect on the IGF system and attenuating IGF-independent growth inhibitory effects.

The expression of IGFBP3 can be regulated by many factors. The pure antiestrogen ICI 182,780 increases IGFBP-3 expression in conditioned medium of MCF7 cells (Pratt and Pollak 1993). Hypermethylation of the IGFBP-3 promoter can down-regulate IGFBP-3 synthesis, resulting in stimulation of the IGF response in non-small cell lung cancer, and is associated with poor prognosis (Chang, Kong et al. 2002a; Chang, Wang et al. 2002b). Given the multiple inhibitory effects of IGFBP3 on the promotion of many cancers, agents that stimulate expression of IGFBP-3, such as anti-oestrogens, anti-androgens, and retinoic acid analogues, may have an anti-tumour effect *in vitro* and *in vivo* (Baserga 1996). Therefore, these agents, together with administration of IGFBP-3 itself, may be potent anticancer agents in future clinical treatments.

1.4.2 Fibronectin

FN1 is a high-molecular-weight adhesive glycoprotein that is involved in cellular adhesion and migration processes including embryogenesis, wound healing, blood coagulation and metastasis. It presents in the extracellular matrix (ECM) as an

insoluble form and in body fluids as a soluble form. The insoluble molecule is widely distributed in healthy connective tissue, in the lamina propria, in vessel structures, nerves and smooth-muscle cell layers (Waalkes, Atschekzei et al. 2010). A single gene encodes FN1 but alternative splicing of pre-mRNA as well as post-translational modifications produce up to 20 variant isoforms in humans (ffrench-Constant 1995). FN1 binds to a number of important molecules, including integrins, heparin, collagen and fibrin, among which integrins are regarded most important since they are structurally and functionally related cell-surface heterodimeric receptors that link the ECM with the intracellular cytoskeleton (Pankov and Yamada 2002).

There are many reports about the involvement of FN1 in different cancers but its function varies among these cancers. For example, over expression of FN1 protein is found in hepatocellular carcinoma (Torbenson, Wang et al. 2002). FN1 is reported to be related to the twist-(EMT inducer), regulating cell motility and invasion in gastric cancer cell lines (Yang, Zhang et al. 2007). Moreover, FN1 promotes invasion of the colorectal cancer cell line Colo320 (Ding, Li et al. 2008) and tyrosine phosphorylation of paxillin together with cell invasiveness in the gastric cancer cell line AGS (Li, Ding et al. 2009). Oxidative stress that accompanies senescence may stimulate FN1 produced by human omentum-derived peritoneal mesothelial cells (HOMCs) and thus promote binding and dissemination of ovarian cancer cells (Ksiazek, Mikula-Pietrasik et al. 2009). In contrast, FN1 strongly decreases invasion of ovarian cancer cell line SKOV-3 in a matrigel transfilter assay (Zand, Qiang et al. 2003). Lastly, FN1 mediates activation of Akt2 which protects human ovarian and breast cancer cells from apoptosis induced by docetaxel via inhibition of the p38 pathway (Xing, Cao et al. 2008).

FN1 can be regulated by oestrogen. E_2 inhibits FN1 expression in the breast cancer cell line MCF-7 *in vitro* and FN1 expression in the tumours of E_2- treated mice is lower than that of control mice *in vivo* (Horii, Takei et al. 2006). In RUCA-I rat endometrial adenocarcinoma cells, the formation and secretion of FN1 is inhibited by E_2 and induced by the anti-oestrogen ICI 164, 384 (Vollmer, Hopert et al. 1995).

Moreover, FN1 mRNA expression is significantly stimulated by E_2 in PEO-1ovarian cancer cell line cells (O'Donnell, Macleod et al. 2005).

1.4.3 Lysyl oxidase

LOX is a secreted, copper-dependent amine oxidase, which catalyzes the production of aldehydes from lysine residues in collagen and elastin precursors. The subsequent condensation of the aldehydes forms the cross-links between collagen and elastin monomers in the ECM (Smith-Mungo and Kagan 1998). The cross-links created by LOX lead to an increased tensile strength and structural integrity, and are essential for normal connective tissue function, embryonic development, and wound healing (Casey and MacDonald 1997). Therefore, aberrant LOX expression or enzymatic activity causes diseases. In fibrotic disease of the lung and atherosclerosis, LOX activity is found to be stimulated.

In addition to its action in ECM, there is evidence suggesting LOX may be a tumour regulator, involved in the development of different cancers. The expression of LOX is reduced progressively with the transition from normal prostate epithelium to malignant prostate epithelium (Ren, Yang et al. 1998). Decreased LOX levels have also been reported in colon tumours (Csiszar, Fong et al. 2002), ductal breast carcinoma cells (Peyrol, Raccurt et al. 1997) and bronchogenic carcinoma (Woznick, Braddock et al. 2005). Paradoxically, it has also been shown that LOX plays a key role in hypoxia-induced metastasis. Studies show that LOX expression is enhanced by hypoxia-inducible factor (HIF) and is associated with hypoxia related metastasis in human breast, head and neck tumours (Erler, Bennewith et al. 2006).

There is evidence that the enzymic activity of LOX can be regulated by oestrogen. In one study, LOX was significantly activated by oestrogen in both skin and bone of mice, suggesting that oestrogen stimulates LOX activity and maturation of collagen and elastin in the extracellular space (Sanada, Shikata et al. 1978). In another study, LOX activity in the mouse cervix was raised after the injection of 1µg E_2 for 10 days following ovariectomy, indicating that LOX activity in the mouse cervix is under the control of oestrogen (Ozasa, Tominaga et al. 1981). Taken together, the above data

indicate that LOX is a cancer-associated and oestrogen-regulated gene that may play an important role in tumour development, deserving further investigation of its effect in cancer.

1.4.4 E-cadherin

E-cadherin, encoded by CDH1 gene and a member of the cadherin super family, is a calcium-dependent cell-cell adhesion membrane glycoprotein. It comprises five extracellular cadherin repeats in the extracellular domain, a transmembrane region, and an intracellular domain that binds catenin. It mediates calcium-dependent homophilic interactions, as the major adhesion receptor in adherens junctions, which is vital to establish and maintain cell-cell junctions (Takeichi 1995; Aberle, Schwartz et al. 1996). Intracellularly, E-cadherin is connected to the catenins, which link E-cadherin to the actin cytoskeleton. Failure either to assemble the E-cadherin/catenin complex or to connect to the actin cytoskeleton correctly leads to the loss of cell adhesion (Semb and Christofori 1998).

Apart from the role in cell–cell adhesion, E-cadherin also functions as a tumour suppressor protein. Loss of function is thought to contribute to progression in cancer by increasing proliferation, invasion, and/or metastasis. It has been reported that some types of tumour lose E-cadherin expression partially or completely as they develop towards malignancy (Birchmeier and Behrens 1994). Ovarian cancer cells with low E-cadherin expression are more invasive (Veatch, Carson et al. 1994), and the absence of E-cadherin expression in ovarian cancers demonstrates poor patient survival in comparison to ovarian tumours with E-cadherin expression (Darai, Scoazec et al. 1997). Furthermore, the reestablishment of a functional cadherin reverses invasive tumour cells to a benign, epithelial cellular phenotype (Vleminckx, Vakaet et al. 1991; Birchmeier and Behrens 1994), indicating the suppressive function of E-cadherin in tumour progression.

There is evidence that oestrogen can regulate the expression and action of E-cadherin. Thus, E_2 suppresses E-cadherin expression by stimulating the expression of the transcription factors, Snail and Slug in an ovarian cancer cell line (Park, Cheung et al.

2008). Moreover, E_2 down-regulates E-cadherin in both normal and tumourigenic breast epithelial cells (Oesterreich, Deng et al. 2003). It is therefore likely that E-cadherin is also a cancer-associated and oestrogen-regulated gene and its action in ovarian cancer needs to be further studied.

1.5 Hypotheses and aims

The hypotheses of the thesis are:

1. There is increasing evidence showing that oestrogens are involved in the development of ovarian cancer. Given that EOC frequently occurs in post-menopausal women, it is hypothesised the oestrogen producing and metabolizing enzymes exist in EOC facilitating local production of E_2, the active oestrogen.
2. Inflammation is involved in the development of EOC, thus it is hypothesised that inflammatory cytokines promote oestrogen biosynthesis in EOC.
3. ERT users have higher risk of EOC, thus it is hypothesised exogenous CEEs can be converted to active oestrogens locally, playing an important role in the development of EOC by regulating oestrogen-responsive genes.

Therefore, the main aims of the studies in this thesis are:

1. To investigate oestrogen signalling in OSE and EOC by studying expression patterns of oestrogen producing and metabolizing enzymes and ERs in OSE and EOC.

2. To investigate the STS/EST pathway further by studying the regulation of the oestrogen metabolizing enzymes by ovarian-associated inflammatory cytokines, specifically IL-1α, in OSE and EOC *in vitro*.

3. To investigate the effect of ERT on EOC by studying the regulation of cancer-associated genes: FN1, IGFBP3, LOX and E-cadherin by ingredients of the ERT preparation, Premarin.

Chapter 2

Subjects and methods

2.1 Clinical tissue resource and collection

2.1.1 OSE cells and normal ovary tissue

Normal OSE cells for *in vitro* experiments were collected from patients undergoing surgery for benign gynaecological diseases, such as fibroids, heavy menstruation bleeding, pelvic pain and infertility. All the patients were of reproductive age and pre-menopausal. Exclusion criteria included malignancy, current pelvic inflammatory disease, endometriosis and use of gonadotrophin-releasing hormone agonists or antagonists. While the use of hormonal contraception was not an exclusion criterion, information of exogenous hormone use was documented in patient files. All samples were allocated a research number and were then anonymised. Before the surgery, formal written consent was obtained from all patients with local ethics committee approval (reference number S1103/36, Chief Investigator Dr K S Fegan). Individual details of each patient used for the project are listed in the relevant chapters.

Normal ovarian tissues for histological studies were obtained from two sources. Some pre-menopausal and post-menopausal ovaries were collected from patients with benign gynaecological disease who had their ovaries removed during surgery. The inclusion and exclusion criteria were similar to those for the collection of OSE cells stated above. The rest of the ovaries were kindly provided by Dr Alistair Williams, University of Edinburgh. Basic clinical information for every patient is given in the relevant chapters.

2.1.2 Ovarian cancer tissue

Ovarian cancer tissues were donated by patients who were suspected of having ovarian cancer and attended for initial laparotomy. They were asked to donate tissue for research in accordance with good clinical practice guidelines. The study was approved by the local ethics research committee (reference number S1103/44, Chief Investigator Dr K S Fegan) and formal written consent was obtained from all the patients. Clinical information for each patient is given in the appropriate chapter. During surgery the excised specimens were placed in sterile polythene bags and transferred to the pathology laboratory for specimen dissection by a consultant

pathologist. This step ensured that the sample represented real tumour tissue and avoided interference with the final pathology result. The obtained samples were placed immediately in a sterile universal container containing 20ml PBS (Gibco, Paisley, Scotland, UK) to obtain EOC cells by enzyme digestion or neutral buffered formalin (NBF; Sigma-Aldrich, Dorset, UK) for histological studies, placed on ice and transferred to the laboratory.

2.2 Cell culture

2.2.1 Establishment of cell monolayers

OSE cells were collected by gently scraping the ovary at the start of surgery with a sterile wooden spatula at laparotomy or a Tao cytobrush (Cook, Ireland Ltd. Limerick, Ireland) at laparoscopy. The collecting instruments were rinsed into 15ml pre-warmed HOSE I medium containing Medium 199:MCDB 105 (1:1, v/v; Invitrogen) supplemented with 15% (v/v) fetal bovine serum (FBS), 50IU/ml penicillin, 50ug/ml streptomycin and 2mM L-glutamine (Sigma-Aldrich) to dislodge the cells, which were then transferred to the laboratory at room temperature. The cells suspension was then transferred into a 75 cm^2 flask (Corning, BV Life Sciences, Amsterdam, the Netherlands) and incubated in a humidified tissue culture incubator gassed with 95% air:5% CO_2 at 37℃ for 7 days to allow cell attachment. The cultures were inspected weekly, with a medium change until cell confluence was reached. Cells were routinely examined by phase-contrast microscopy for contaminating cells such as fibroblasts. If flasks contained more than 10% contamination with fibroblast-like cells, the flasks would be discarded and not used for later experiments. Once reaching confluence, usually between 2 to 4 weeks, cells were used for the experiments or passaged with a split ratio of 1:3 into 3 new 75 cm^2 flasks to grow until confluence again for later use. All the OSE cells used for the project were within 3 passages.

EOC cells from ovarian cancer tissue were kind gifts from Dr K S Fegan and the method to collect these cells was as follows. In a Class 2 tissue culture hood, the tumour was placed in a 5cm diameter Petri dish and minced using two sterile scalpel

blades. The minced tumour was then transferred to a 50ml Falcon tube (Corning) for enzymatic tissue digestion. The 'digestion mix' was 0.25% trypsin (Gibco), 0.004% DNAseI (Sigma-Aldrich) in 20ml cold HOSE II medium, which was similar to HOSE I medium but with 0.01% (w/v) bovine serum albumin (BSA; Sigma-Aldrich) instead of FBS to prevent denaturing of enzyme activity. The digest was left mixing on a shaker overnight at 4°C. The following morning the solid components were allowed to settle and the supernatant aspirated, combined with HOSE I medium to inactivate the trypsin and the cells were collected by centrifugation at 500×g for 5 minutes. The cell pellet was resuspended in 15ml HOSE I medium and placed in a $75cm^2$ tissue culture flask. The remaining minced tumour was mixed with another 20ml digestion mix and placed in an agitated water bath at 37°C for 30min. The supernatant fluid was then aspirated, inactivated with an equivalent volume HOSE I medium and centrifuged as above to form a pellet which was then resuspended in 15ml fresh HOSE I medium and transferred to a second $75cm^2$ tissue culture flask. The tumour mince was washed and pelleted two more times so that four flasks were obtained from every piece of solid tumour. Flasks were left in an atmosphere of 5% CO2, 95% air at 37°C for one week to allow cells to attach. HOSE I medium was then changed twice weekly until confluence was reached.

SKOV-3 and ***PEO-14 cell lines*** were kindly provided by P Pujol (INSERM, Montpellier, France) and ***PEO-1 cell line*** was a gift from Dr Simon Langdon (Cancer Research UK, Edinburgh Oncology Unit, Edinburgh, UK). The cells were grown in 162 cm^2 flasks (Corning) at 37°C, in an atmosphere of 95% air and 5% $CO_{2,}$, with similar HOSE I medium to OSE cells but containing 10% instead of 15% FBS. Culture medium was changed twice per week and cells were split 1:2 or 1:3 when they were confluent. Once the cells had been passaged 20 times, they were discarded and cells which had been frozen at the same initial passage number and in the same batch were defrosted and cultured instead.

2.2.2 Experimental treatments

After the cell monolayer had reached confluence, culture medium was removed and the cells were washed with 10ml Dulbecco's phosphate buffered saline (DPBS; Sigma-Aldrich) twice and incubated in 5ml (75cm^2 flasks) or 10ml (162 cm^2 flasks) Trypsin/EDTA (0.05%, w/v trypsin, 0.5mM EDTA; Invitrogen) solution at 37°C for five minutes. After all the cells had detached from the flask, trypsin was neutralised with an equal volume of HOSE I medium. The cell suspension was transferred to 50mL centrifuge tubes (Corning) and the cells were then collected by centrifugation at 800×g for 3 minutes, with the supernatant being discarded. The cell pellet was re-suspended in 5ml pre-warmed fresh HOSE I medium, and the cell number was counted using a haemocytometer. Cell viability was assessed by Trypan Blue exclusion (Sigma-Aldrich): the viability of OSE cells and cell lines was between 80% and 95%. The dispersed cells were plated into 6-well culture plates (Corning) with 300,000 cells per well for mRNA studies or 500,000 cells per well for enzyme activity studies. For *in vitro* scratch assay studies the suspended cells were seeded on 12-well culture plates (Corning) with 150,000 cells per well. After incubation in the same environment used above for 24h, the cells formed monolayers. The medium was then discarded and cells were washed with DPBS twice, followed by culture in HOSE II culture medium for 24h. Cells were then treated with the factors of interest. Factor concentrations and incubation times are described in the relevant experimental chapters.

2.3 Histological staining

Expression patterns and localisation of proteins in the ovary and ovarian cancers were investigated by immunohistochemistry using a number of specific primary antibodies. A standard streptavidin-biotin horseradish peroxidase (HRP) method was employed. Briefly, the primary antibody binds specifically to the protein of interest, and the biotinylated secondary recognises and binds to antigenic sites on the primary antibody. HRP labelled streptavidin binds to the biotin on the secondary antibody and DAB binds the HRP and is oxidised. This causes a visible colour change, which results in the brown staining of tissue sections where the antibody has bound.

2.3.1 Tissue processing

Tissue samples for histology were collected in neutral buffered formalin (NBF) (Sigma-Aldrich) and fixed at 4°C for 24h, then transferred to 70% ethanol and stored at room temperature prior to wax embedding which was carried out in the Human Reproductive Sciences Unit (HRSU) Histology facility. Samples were placed in labelled cassettes in 70% ethanol for automatic processing. Using a Leica TP1050 automatic processor, samples underwent dehydration in graded alcohols for 1.5h in each alcohol, 2x2h in absolute alcohol, then 3x3h in xylene, before going through three chambers of molten wax for 1, 1, and 1.5 h respectively. Samples were then aligned correctly in metal moulds, and molten paraffin wax poured into each mould. The cassette was placed on top of the molten wax and the mould placed on a cooling block to set. When set, the mould could be gently eased off. Embedded tissue sections were stored at room temperature. When required, the blocks were cooled on ice to make the blocks easier to cut and 5µm sections were cut by a hand-operated microtome (Leica Microsystems, Milton Keynes, UK). Sections were floated on distilled water at 37°C in a heated waterbath and gently picked up onto charged coated slides. Sections were dried overnight at 50°C in a drying oven (Lamb RA, East Sussex, UK) then stored at room temperature until used.

2.3.2 Dewaxing and rehydration

Slides were appropriately labelled, dewaxed in xylene for 10min (2x5min), then rehydrated in graded alcohols [100%, 100%, 95% (v/v), 70% (v/v), 50% (v/v)] for 20sec each. Slides were washed in distilled water for 10min.

2.3.3 Antigen Retrieval

Antigen retrieval was performed by pressure cooking in 10mM sodium citrate buffer (pH 6.0). 2L of buffer was placed inside a pressure cooker on an electronic plate heater and allowed to heat. When the buffer was almost boiling, the slides were gently placed inside the pressure cooker, ensuring they were completely immersed in buffer and the lid sealed. When at full pressure which was indicated by steady hissing, the slides were heated for 5min, then the cooker was moved away from the heater immediately and pressure was released slowly. After the sections cooled in the

citrate buffer for 20min, the lid was removed and the sections were allowed to cool further by slowly mixing tap water with the buffer. Following cooling, the sections were washed in distilled water twice for 10min.

2.3.4 Blocking procedures

Endogenous peroxidases were blocked to prevent excess background staining. A 300mL 3% hydrogen peroxide solution was made by diluting 30mL 30% stock hydrogen peroxide (VWR International Ltd, Lutterworth, Leicestershire, UK) in 270mL pure methanol (Fisher Scientific, Loughborough, Leicestershire, UK). Slides were immersed in the solution for 30min at room temperature, and then washed in 50mM Tris buffered saline (TBS) for 2x5min. The slides were agitated gently on a rocker during the whole procedure to ensure thorough blocking and washing. Background staining was further prevented by the inclusion of a non-immune serum block to prevent non-specific binding of the secondary antibody. A blocking solution was prepared by diluting non-immune serum of the species in which the secondary antibody had been raised 1:5 (v/v) in TBS containing 5% (w/v) BSA (Sigma-Aldrich). This solution was applied to slides in a humidified chamber and incubated for 20min at room temperature. There was no wash following this step. Endogenous streptavidin and biotin also need to be blocked to prevent excess background staining. A streptavidin-biotin blocking kit (Vector Laboratories Ltd, Peterborough, UK) was used. The slides were put back into the humidified chamber and one or two drops of streptavidin were applied on every slide, depending on the size of the section. The slides were then incubated for 15min followed by washing in TBS for 2x5min with agitation and returning to the humidified chamber where one or two drops of biotin were applied to each slide. Sections were incubated at room temperature for a further 15min, and then washed again in TBS for 2x5min with agitation.

2.3.5 Primary antibody

Primary antibodies were diluted to the appropriate concentration in the serum used for blocking. The primary antibodies used and the working dilutions are shown in Table 2.1 below. Generic immunoglobulins from the same species, or green

fluorescent protein (GFP) antibody raised in the same species at the same concentration as the primary antibody, were used as a negative control. Positive controls were tissue sections known to express high levels of the protein of interest. Details of specific positive and negative controls are given in Table 2.1 below. Primary antibodies and negative controls were applied to sections in a humidified chamber to prevent drying. Sections were incubated overnight (approx. 18h) at 4°C, followed by washes in TBS for 2x5min with gentle agitation.

Primary antibody	Dilution	Host species	Secondary antibody	Source	Positive control	Negative control
STS	1:75	Rabbit	Goat anti-rabbit (Dako, Cambridge, UK)	Sigma-Aldrich (HPA002904)	Placenta	Rabbit IgG
EST	1:750	Rabbit anti mouse	Goat anti-rabbit	Gift 1	Fetus Kidney	GFP antibody raised in rabbit
Aromatase	1:50	Mouse	Goat anti-mouse (Dako, Cambridge, UK)	Gift 2	Placenta	Mouse IgG
17βHSD2	1:200	Rabbit	Goat anti-rabbit	Gift 3	Placenta	GFP antibody raised in rabbit
17βHSD5	1:150	Mouse	Goat anti-mouse	Gift 4	Endometrium	Mouse IgG

Table 2. 1: Primary antibodies used in the project, which were purchased (HPA0020904) or generously donated, as follows.

Gift 1: Prof. Masahiko Negishi (Song, Qian et al. 1997)

Gift 2: Prof. WR Miller (Sasano, Anderson et al. 2005)

Gift 3: Prof. Yves Tremblay (Drolet, Simard et al. 2007)

Gift 4: Prof. Trevor Penning (Lin, Steckelbroeck et al. 2004)

2.3.6 Secondary antibodies

Biotinylated secondary antibodies (Dako, Cambridge, UK) were diluted 1:500 in the serum solutions used for the non-immune block. The antibody was applied to sections in a humidified chamber and incubated for 1 hour at room temperature. Following incubation slides were washed in TBS for 2x5min with gentle agitation.

2.3.7 Antigen detection

Streptavidin HRP was used to amplify the signal from the biotinylated secondary antibody. The streptavidin HRP solution was prepared by adding 1µl streptavidin HRP into 1000µl TBS. The solution was added to the slides in a humidified chamber, incubated for 30min at room temperature, and then washed for 2x5min in TBS with gentle agitation. Slides were developed and stained with 3,3-diaminobenzidine (DAB; Dako). One drop of DAB solution was added to 1ml of DAB buffer and two drops of DAB mix were added onto sections in a humidified chamber. When brown staining was visualised, DAB was washed off with copious distilled water. Sections were then counterstained in haematoxylin (Sigma-Aldrich) for 30sec followed by a wash in running tap-water and in Scott's tap water (Pioneer Research Chemicals Ltd.) for 10sec followed by a wash in running water. Slides were then dehydrated through increasing alcohol concentrations [70% (v/v), 80% (v/v), 95% (v/v), 100%, 100%; 20sec in every concentration] and cleared in xylene for 2x5min before mounting under a glass cover slip (VWR) using Pertex mounting medium (Cellpath Plc, Hemel Hempstead, UK).

2.4 Molecular studies

2.4.1 RNA extraction

2.4.1.1 RNeasy Mini Kit extraction

Total RNA was extracted from cultured cells using the RNeasy Mini Kit (Qiagen Ltd, West Sussex, UK) following the manufacturer's instructions. Culture medium was removed from the wells and 350µl lysis buffer (RLT buffer) supplemented with 1% β-mercaptoethanol (v/v) added directly onto each culture well and repeatedly

pipetted to ensure complete cell lysis. The suspension was transferred to a 1.5ml tube, and frozen at -70°C. Cell lysates were removed from storage and thawed in a waterbath at 37°C for 15min prior to extraction.

350µl 70% ethanol was added to the lysate and mixed by pipetting. The mixture was transferred to an RNeasy mini spin column and centrifuged at 9520×g (Hettich MICRO 200, GMI Inc, Minnesota**,** USA) for 15sec. The flow-through was discarded. The column was then washed with 350µl Buffer RW1 supplied with the kit and centrifuged at 9520×g for 15sec. The flow-through was again discarded. To preclude the presence of genomic DNA that could influence the accuracy of the results, on-column DNase digestion was performed using RNase-Free DNase set (Qiagen). DNase digestion was performed by diluting DNase I 1:7 (v/v) in Buffer RDD, adding 80µl of the mixture to each column and incubating at room temperature for 15min. DNase was removed by washing with 350µl Buffer RW1 and centrifuging at 9520×g for 15sec. Two more washes were performed with 500µl Buffer RPE, centrifuged at 9520×g for 15sec and 2min respectively. The flow-through was discarded after each contrifugation. The RNeasy column was then put in a new 2ml tube and centrifuged at 18700×g for 1min to dry the column. The column was then transferred to a fresh RNase-free 1.5ml collection tube, with 30µl of RNase-free water added directly to the column membrane to elute the extracted RNA. After centrifuging at 9520×g for 1min, the eluate was removed from the tube and passed through the column once again followed by centrifuging at 9520×g for 1min to obtain a higher concentration of RNA. The RNA in solution was stored at -70°C.

2.4.1.2 Measuring RNA concentration and quality

The RNA was quantified using the Nanodrop-1000 Spectrophotometer (Nanodrop Technologies Inc, Wilmington, DE, USA). A 260/280 ratio above 2.0 indicates no detectable protein contamination. Only RNA samples with ratios above 2.0 were used for cDNA synthesis. Samples were stored at -80°C until further use.

2.4.2 Reverse transcription

Reverse transcription was conducted on samples with a suitable RNA yield using a High Capacity complementary DNA (cDNA) Reverse Transcriptase Kit (Applied Biosystems, Warrington, UK). 3.4µl of reagent mixture (outlined in Table 2.2), 200ng RNA and RNase-free water made a 10µl cDNA reaction mixture. Two negative controls were also included, a "RT-negative" containing no MultiScribe Reverse Transcriptase and a "RT-water" containing nuclease-free water in place of RNA. The reaction mixtures were thoroughly vortexed and briefly centrifuged in a micro centrifuge to ensure all contents were at the bottom of the tube. The tubes were loaded into a GRI G-storm Thermal cycler (GRI, Braintree, Essex, UK) and incubated for 25°C for 10 minutes (annealing of random hexamers), 37°C for 120 minutes (reverse transcription), 85°C for 5 minutes (denaturation of RNA-cDNA double strand and RNA degradation) and 4°C on hold cycle. The cDNA was then stored at -20°C until required for quantitative PCR analysis.

Reagent	**Reagent concentration**	**Volume per reaction (µl)**
10x RT Buffer	10x	1.0
10x RT Random Primers	5µM	1.0
25x dNTP Mix	4mM	0.4
Multiscribe Reverse Transcriptase	2.5U/µl	0.5
RNase Inhibitor	1U/µl	0.5

Table 2.2: Reagents for reverse transcription. Volumes shown are for one reaction when combined with RNA (200ng) with RNase-free water to give a total reaction volume of 10µl.

2.4.3 Quantitative Real-Time polymerase chain reaction (PCR)

Quantitative Real-Time PCR (qRT-PCR) measures levels of a gene-specific sequence of cDNA generated in a reverse-transcriptase PCR reaction, and thus provides a measure of RNA expression in a tissue. QRT-PCR was carried out using the Taqman system and ABI Prism 7900HT sequence detection system (Applied Biosystems). This works by producing a fluorescent signal during the amplification

of the PCR product. Forward and reverse primers are designed for a target DNA sequence and the probes are 20-30bp oligonucleotides that are complementary to a sequence flanked by the forward and reverse primers of the target genes. The probe has two labels, a reporter label at the 5' end (FAM; 6-carboxyfluorosceine) and a quencher at the 3' end (TAMRA; 6-carboxytetramethylrhodamine). In each reaction, when the probe is intact, the quencher suppresses the fluorescence of the reporter primarily by Forster-type energy transfer (Forster, 1948; Lakowicz, 1983). During PCR, if the target cDNA of interest is present, the probe specifically anneals between the forward and reverse primer sites. The 5' to 3' nucleolytic activity of the AmpliTaq Gold polymerase (within Taqman Universal PCR Master Mix, PE Applied Biosystems) cleaves the 5'- end of the probe and the reporter and quencher are separated. Fluorescence can now be detected. The amount of fluorescence is directly proportional to the amount of PCR product, and non-specific amplification does not cause fluorescence (Taqman PCR Reagent Kit Protocol, Applied Biosystems). This is represented diagrammatically in Figure 2.1.

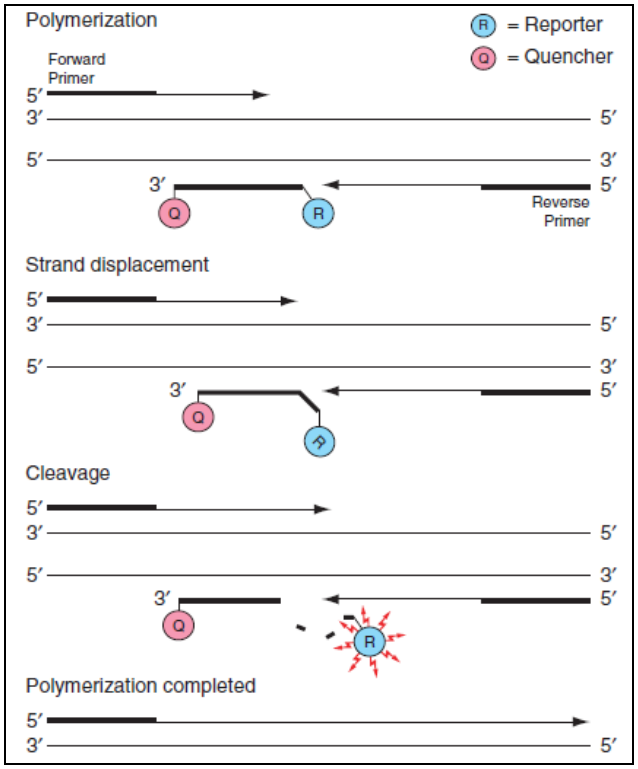

Figure 2.1: Diagrammatic representation of Taqman qRT-PCR. Adapted from Taqman PCR Reagent Kit Protocol (Applied Biosystems).

For the relative quantification of the results, 18S ribosomal RNA detection is used as an internal control in this system. The 18S probe also has a reporter (VIC), which emits fluorescence at a different wavelength to the FAM reporter on the gene of interest probe. Quantification is relative to 18S since expression levels remain relatively constant in cells under different conditions.

Taqman primers and probes of target genes were either designed in-house by ProbeFinder version 2.45 (Roche Diagnostics Ltd., Burgess Hill, UK) or by Primer Express software or purchased as Assay on Demand (Applied Biosystems). Probes designed in-house by ProbeFinder (EST, FN1, IGFBP3 and E-cadherin) were selected from the Universal Probe Library (Roche Diagnostics Ltd.) and primers that matched the probes were synthesized by Genosys Biotechnologies (Cambridge, UK)

and diluted to 250nM in nuclease-free water (Sigma). Primers designed in-house by Primer Express software (ERα, ERβ and 17βHSD2) were manufactured by Biosource (Nivelles, Belgium) or Eurogentec (Southampton, UK) and diluted to 250nM, and probes to 50nM in nuclease-free water (Sigma). Probes and primers were all validated before use. Assay on Demand primer/probe sets were used directly in the reactions as they were provided pre-optimised at a final concentration of 900nM primer and 250µM probe and primer/probe sets were pre-validated by ABI. Details of primers and probes used are given in Table 2.3.

Gene	**FW Primer (5'-3')**	**RV Primer (5'-3')**	**Probe (5'FAM-TAMRA/MGB 3')**	**NCBI accession or reference number**	**Positive control**
STS	Assay-on-demand	Assay-on-demand	Assay-on-demand	Hs00165853_m1	placenta
EST	AAGACTC ATTTGCC ACCTGAA	GCATTCC GGCAAAG ATAGAT	Roche Probe library number: 4	NM_005420.2	endometrium
Aromatase	Assay-on-demand	Assay-on-demand	Assay-on-demand	Hs00240671_m1	placenta
17βHSD1	Assay-on-demand	Assay-on-demand	Assay-on-demand	Hs00166219_m1	placenta
17βHSD2	TGTCAGC AGCATGG GAGGA	GGTCACA GCCGCCT TTGAT	CCCCAAT GGAAAGG CTGGCAT CTT	NM_002153.2	endometrium
17βHSD5	Assay-on-demand	Assay-on-demand	Assay-on-demand	Hs00366267_m1	placenta
ERα	TGATTGG TCTCGTCT GGCG	CATGCCC TCTACAC ATTTTCCC	TGCTCCTA ACTTGCTC TTGGACA GGAACC	NM_000125.3	placenta

Gene	FW Primer (5'-3')	RV Primer (5'-3')	Probe (5'FAM-TAMRA/MGB 3')	NCBI accession or reference number	Positive control
ERβ	GGTCCATCGCCAGTTATCACAT	GATGCGTAATCGCTGCAGACAG	TGTGAAGCAAGATCGCTAGAACACACCT	NM_001437.2	placenta
OATP-B	Assay-on-demand	Assay-on-demand	Assay-on-demand	Hs00200670_m1	placenta
OATP-D	Assay-on-demand	Assay-on-demand	Assay-on-demand	Hs00203184_m1	placenta
OATP-E	Assay-on-demand	Assay-on-demand	Assay-on-demand	Hs00249583_m1	placenta
OAT4	Assay-on-demand	Assay-on-demand	Assay-on-demand	Hs00218486_m1	placenta
FN1	CTGGCCGAAAATACATTGTAAA	CCACAGTCGGGTCAGGAG	Probe number: 32	NM_002026.2	placenta
IGFBP3	AACGCTAGTGCCGTCAGC	CGGTCTTCCTCCGACTCAC	Probe number: 1	NM_000598.4 NM_001013398.1	placenta
LOX	Assay-on-demand	Assay-on-demand	Assay-on-demand	Hs00184700_m1	ovary
E-cadherin	CCCGGGACAACGTTTATTAC	GCTGGCTCAAGTCAAAGTCC	Probe number: 35	NM_004360.3	placenta

Table 2.3: Sequences of primer/probes sets and positive control of every gene for qRT-PCR.
FW: forward, RV: reverse
Sequences of assay-on-demand primers and probes were unavailable but were pre-validated by ABI.
Probe number referred to the number of the probe in the Universal Probe Library and sequences were unavailable.

0.8µl or 2µl cDNA was analyzed in a 10µl or 25µl reaction mixtures respectively. Each reaction contained the following reagents (final concentration): 1) 1x TaqMan Universal PCR Master Mix which included 10x PCR buffer, 5.5mM $MgCl_2$, 200µM dNTPs (400µM dUTP), 1.25U/µl AmpliTaq DNA Polymerase and Uracil-N-glycosylase (UNG) (Applied Biosystems); 2) 50nM 18s primers and probes (Applied Biosystems); 3) specific primers and probes of target genes and 4) nuclease-free water up to 10 or 23µl. All samples were run in duplicate or triplicate in either 384-well (for 10µl system) or 96-well plates (for 25µl system) (Applied Biosystems). Three negative controls were included on each plate, 1) negative reverse transcribed sample (RNA sample no reverse transcriptase enzyme); 2) reverse transcribed H_2O (water in place of mRNA sample); and 3) TaqMan reaction negative control (cDNA replaced with H_2O). Suitable positive controls used for every target genes are listed in Table 2.3. Wells were sealed using an optical cover (Applied Biosystems) and Taqman reactions were carried out in an ABI Prism 7900 Sequence Detector (Applied Biosystems) following the default protocol (Table 2.4, Dorak 2004, Applied Biosystems Manual):

Step	**Programme**	**Function**
1	50°C for 2 minutes	Activation of UNG which prevents reamplification of carry-over PCR products by removing any uracil incorporated into amplicons (Dorak, 2004)
2	95°C for 10 minutes	Activation of the AmpliTaq enzyme
3	40 cycles of: 95°C for 15 seconds 60°C for 1 minute	 Denaturation Annealing and extension

Table 2.4: Protocol for Taqman qRT-PCR.

Data were acquired when PCR amplification was in the exponential phase. The data output are in the form of the cycle number where the fluorescence of the reporter dye rises above a threshold, this value is the threshold cycle, CT. A CT value was obtained for both the gene of interest and 18S. These data were transferred to a Microsoft Excel spreadsheet for analysis. When the CT value for the mRNA was more than 38, the mRNA expression of the gene in the sample was regarded undetectable. There were four calculations performed on the data. Firstly, the CT value for the mRNA of the gene of interest was normalised to the CT value for the 18S ribosomal RNA used as an internal control, by subtracting the 18S CT value from the CT value of the gene of interest, which equals the ΔCT value. As each sample was run in duplicate or triplicate, the average ΔCT was then calculated as the mean of the 2 or 3 ΔCT values for each sample. The next step was the calculation of ΔΔCT by subtracting the ΔCT of a comparator which varied in different experiments from the average ΔCT of each sample. The comparators for every experiment were denoted in relevant chapters. The final calculation was to get [2^ (- ΔΔCT)] value for each sample, to give a fold increase in expression over comparator. The mean fold increase (or decrease) for each sample group was then calculated and compared.

2.5 Enzyme activity assays

As described above, cells in the 6-well plates (500, 000 cells/well) were prepared for the assays. The following morning the culture medium was changed to serum-free HOSE II medium and incubated for a further 24h. On the third day culture medium was removed from the wells and replaced with treatments in fresh HOSE II medium. The medium contained 3nM E_1S and 150,000cpm/µl [6,7-^{3}H(N)]-E_1S (PerkinElmer, USA) or 3nM E_1 and 150,000cpm/µl [2,4,6,7-^{3}H]-E_1 (PerkinElmer, USA), depending on the experiments. Following incubation for the desired time, medium was collected into 15ml glass centrifuge tubes and mixed well with 10ml dichloromethane (Fisher Scientific) to stop the reaction and extract steroids. Samples were then centrifuged at 800×g for 15min, the aqueous phase was discarded and the organic phase evaporated to dryness under a nitrogen stream with the steroids remaining in the solvent phase concentrated at the same time. The dry steroid extract was then reconstituted in 100µl fresh dichloromethane with unlabelled E_1 (10µM), E_2 (10µM) and E_1S (10µM)

as carriers for the labelled steroid. Following adequate vortexing, samples were spotted onto silica-gel pre-coated sheets (PE, SILG; Whatman, Maidston, Kent, UK) and run for 1.5h in a sealed tank with chloroform:ethanol (92:8, v/v) as the mobile phase, which separated the steroid components. Finally, the thin layer chromatography (TLC) sheet was taken out of the tank, dried and the radio-labelled steroid was identified on the chromatogram and quantified using a Bioscan 200 imaging detector (Lablogic Systems, Sheffield, UK). The data output is in the forms of region counts. See the Fig.2.2 and Table 2.5 below.

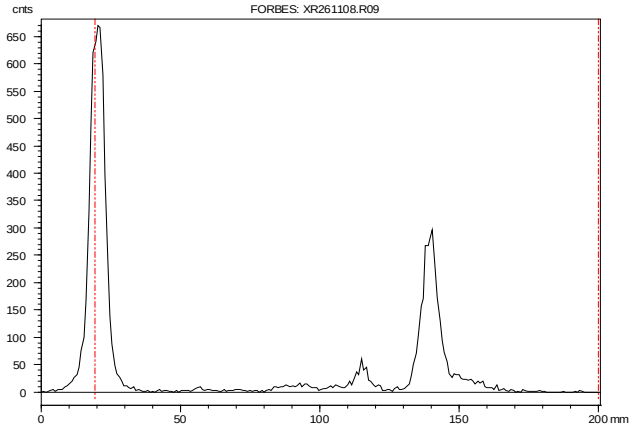

Figure 2.2: Readout from TLC plates where y-axis shows counts of radioactivity and x-axis demonstrates the distance along the plate.

Peak area	Region counts
Peak1	5443
Peak2	324
Peak3	2658

Table 2.5: Data output from Bioscan 200 imaging detector.

The calculation of final conversion of E_1S and E_1 followed the steps below:
Counts of E_1S peak (Peak 1) = X
Counts of E_1 peak (Peak 3) = Y
Counts of E_2 peak (Peak 2) = Z
52.38% recovery for E_1S and 82.76% recovery for E_1 and E_2 (see the explanation below)

A = X/0.5238

B =Y/0.8276

C =Z/0.8276

D (conversion rate of E_1S to E1) = (B+C) / (A+B+C) X 100

E (conversion rate of E_1S to E2) = C / (A+B+C) X 100

Since we started with 2ml of HOSE II medium with 3nM of E_1S, total E_1S is equivalent to 6 pmoles. Therefore conversion of E_1S to E_1 will be D / 100 X 6 / number of hours / number of cells and conversion of E_1S to E_2 will be E / 100 X 6 / number of hours / number of cells. Activity data were computed as fmoles of substrate conversion per hour per 10^5 cells (fmol/h/10^5 cells).

Because the solubilities of E_1S and E_1 or E_2 in dichloramethane are different, the extraction efficiency of E_1S and E_1 or E_2 by dichloramethane is therefore different. A recovery experiment was done to decide the efficiency of extraction. In the recovery experiment, 20ml HOSE II medium was mixed with 3nM E_1S and 150,000cpm/µl [6,7-^{3}H(N)]-E_1S. Mixture was then aliquoted into 6 glass centrifuge tubes with each tube containing 2ml mixture. 3 tubes went through the extraction process stated above while the other 3 tubes did not undergo the extraction. After extraction, the aqueous phase was discarded and the organic phase evaporated to dryness under a nitrogen stream. The dry steroid extract was then redissolved in 2ml HOSE II medium overnight. The total radioactivity in each tube was then measured by adding scintillation fluid, Ecoscint A (National Diagnostics, Hull, England) and counting on a Beta counter, Tri-carb 2100TR (Packard, USA). The extraction efficiency of E_1S was then calculated as follows:

$$\frac{\text{average count of } E_1S \text{ extracted by dicloromethane}}{\text{average count of } E_1S \text{ without extraction}} \times 100\%$$

The extraction efficiency of E_1or E_2 was calculated using the same method as above. The extraction efficiency of E_1S was 52.38% and the extraction efficiency of E_1 or E_2 was 82.76%. The extraction process was carried out in HOSE II medium plus or minus phenol red, and no difference was observed.

2.6 In vitro wound healing assay

PEO-1 cells were grown as a monolayer on 12-well culture plates (Corning) in HOSE I medium for 24h before being changed to phenol-red free medium 199 (Invitrogen) supplemented with charcoal-stripped FBS (10%, v/v), penicillin (50IU/ml), streptomycin (50μg/ml) and L-glutamine (2mmol/l) for a further 24h. The cells were then incubated with E_2 or Eq (10^{-8}M) for 72h, with or without oestrogen antagonist ICI 182, 780 (10^{-6}M), which was added to the above medium one hour before E_2 or Eq treatment. The treatment medium was then removed from the cells and stored at 37°C for later use and replaced with DPBS. The cell monolayer was then scraped gently in a straight line across the full width of the well to create the wound using a p200 pipette tip. To remove excess debris produced by scratching, the cells were then washed twice by DPBS. The saved treatment medium was then replaced into the appropriate treatment wells. The width of the scratch wound was measured using Zeiss Axiovert 5100 Microscope and Openlab software, with measurements made in pixels. Each scratch width was measured at three equally spaced points along its length and treatment groups were measured in triplicate. Cells were incubated in the different treatments for 24h and the distance of each scratch at the same noted point was measured again. Cell migration over 24h was assessed by subtracting the width at 24h from the width measured after scratching, which was called ΔWidth. As each width was measured at three points an average ΔWidth was calculated as the mean of the 3 widths for each wound. The final result was presented as a ratio of ΔWidth of treatment / ΔWidth of the control x 100% and the mean of the ratio for each treatment group was then calculated and compared.

2.7 Statistical analysis

The statistical analyses were variable depending on different experimental methods and numbers of patients or treatment groups and are listed in the relevant chapters.

Chapter 3

Survey of oestrogen metabolizing enzymes in pre-menopausal and post-menopausal ovary and EOC

3.1 Introduction

Oestrogen has been implicated as a causative and promotive factor in many oestrogen-related cancers such as breast and endometrial cancer. Anti-oestrogen therapy has been used as an adjuvant treatment clinically, having benefited patients with ER-positive cancer. Although the question whether ovarian cancer is an oestrogen-dependent disease is still controversial, increasing evidence suggests oestrogen is linked to and promotes the development of ovarian cancer. As described in Chapter 1, some epidemiological data have suggested HRT users have a higher risk of ovarian cancer and this effect is primarily related to the use of oestrogen-only HRT (Lacey, Mink et al. 2002; Lacey, Brinton et al. 2006; Beral, Bull et al. 2007). In addition, oestrogen receptors are expressed in 61%-79% of EOC (Lindgren, Backstrom et al. 2001; Lee, Rosen et al. 2005). Some experimental data demonstrated oestrogen can stimulate the growth of ovarian tumour cell lines that express oestrogen receptors (Langdon, Hawkes et al. 1990). In vivo studies in animals revealed oestrogen accelerates tumour onset and decreases survival in a transgenic mouse model of ovarian cancer (Laviolette, Garson et al. 2010). Furthermore, clinical trials with an aromatase inhibitor (AI), which acts by reducing the levels of oestrogen available to ER, have shown clinical benefit in a sub-group of ovarian cancer patients. Data from Phase II trials have indicated that patients with EOC and ER-positive tumours might benefit from AI therapy (Bowman, Gabra et al. 2002; Smyth, Gourley et al. 2007).

Together these results indicate that ovarian cancer is oestrogen responsive in some, but not all subtypes of ovarian cancer. Paradoxically, ovarian cancer generally occurs in post-menopausal women, in which ovulation has ceased and ovarian E_1 and E_2 production rates are reduced compared to pre-menopause. So the question arises if oestrogen is involved, where does it come from? We hypothesize that the conjugated (inactive) oestrogens circulating in post-menopausal women are substrates for formation of active oestrogen in EOC locally, and that local enzymes can biosynthesize E_2, which in turn promotes the development of ovarian cancer.

The local production of oestrogen is mediated by a number of enzymes. Aromatase catalyzes conversion of T into E_2 and A into E_1. STS hydrolyzes E_1S to E_1. 17βHSD1 or 17βHSD5 subsequently convert E_1 to E_2. By contrast, 17βHSD2 and EST act in the opposite direction to convert E_2 to E_1 and E_1 to E_1S respectively. Thus the local expression of these enzymes is important in regulating oestrogen metabolism and consequently may have a role in ovarian cancer onset and development.

As described in Chapter 1, OSE cells from which EOC is thought to be derived, can become trapped in an inclusion cyst and subsequently develop into ovarian cancer under the influence of the local environment in or around the inclusion cyst. Therefore to investigate the local production of E_2 and its promotive effect on ovarian cancer, this chapter examines the expression of oestrogen metabolizing enzymes in normal OSE cells, OSE cells lining inclusion cysts and ovarian cancer cells. Since ovarian cancer usually occurs after the menopause, it is also necessary to compare the difference in expression of these genes between pre- and post-menopausal women.

Specifically, the aims of this chapter are to study the protein expression patterns of STS, EST, aromatase, 17βHSD2 and 17βHSD5 using immunohistochemistry (IHC) in human pre-menopausal and post-menopausal ovaries, in epithelial ovarian cancer and in inclusion cysts.

3.2 Materials and methods

3.2.1 Ovary and ovarian cancer tissue

Pre- and post-menopausal ovaries, ovaries containing inclusion cysts and ovarian cancer tissues were collected as described in Sections 2.1.1 and 2.1.2. Briefly, 3 pre-menopausal ovaries, 6 post-menopausal ovaries, 7 ovarian cancers and 1 post-menopausal ovary with a few inclusion cysts were collected. The relevant clinical details of these patients are listed in Table 3.1.

Patient /block number	**Age (years)**	**Reason for surgery**	**Ovary diseases**	**Pre/post menopausal**
1 12408/09	47	Fibroids	No	Pre-menopausal
2 13209/09	45	Fibroids	No	Pre-menopausal
3 1106	29	N/A	No	Pre-menopausal
4 10869/09	63	Endometrial cancer	No	Post-menopausal
5 14716/09	62	Chronic pelvic pain	No	Post-menopausal
6 12192/09	72	Endometrial cancer	No	Post-menopausal
7 13543/09	61	Endometrial cancer	No	Post-menopausal
8 13942/09	64	Endometrial cancer	No	Post-menopausal
9 2877	N/A	N/A	No	Post-menopausal

Patient /block number	Age (years)	Reason for surgery	Ovary diseases	Pre/post menopausal
10 RB06-0114	43	Ovarian cancer	Ovarian cancer: Mixed differentiation carcinoma with mucinous, serous and endometrioid features	Pre-menopausal
11 2006-1841	67	Ovarian cancer	Ovarian cancer: serous carcinoma	Post-menopausal
12 2006-1845	59	Ovarian cancer	Ovarian cancer: serous papillary	Post-menopausal
13 2006-2709	61	Ovarian cancer	Ovarian cancer: endometrioid adenocarcinoma	Post-menopausal
14 2006-2731	88	Ovarian cancer	Ovarian cancer: serous/endometrioid carcinoma	Post-menopausal
15 2006-2733	53	Ovarian cancer	Ovarian cancer: serous carcinoma	Post-menopausal
16 2006-3386	66	Ovarian cancer	Ovarian cancer: endometrioid adenocarcinoma	Post-menopausal
17 19594	65	Endometrial cancer	Inclusion cysts	Post-menopausal

Table 3.1: Patient information for IHC studies.

N/A: not available

3.2.2 IHC

IHC was performed as described in Section 2.3.

3.3 Results

3.3.1 Expression of STS protein in human ovary and ovarian cancer

IHC revealed STS protein expression in pre- and post-menopausal ovaries, inclusion cysts and ovarian cancers. Expression was mainly localised to OSE in normal ovary, epithelium of inclusion cysts and ovarian cancer cells in ovarian cancer, with very low expression seen in the endothelial and stromal cells in some samples and low expression in granulosa and theca cells of one pre-menopausal ovary (data not shown). There was no difference in expression patterns between pre-menopausal and post-menopausal ovaries. Intensity of expression did not differ among pre-menopausal OSE, post-menopausal OSE, OSE embedded in inclusion cysts and ovarian cancer cells. These data are shown in Figure 3.1.

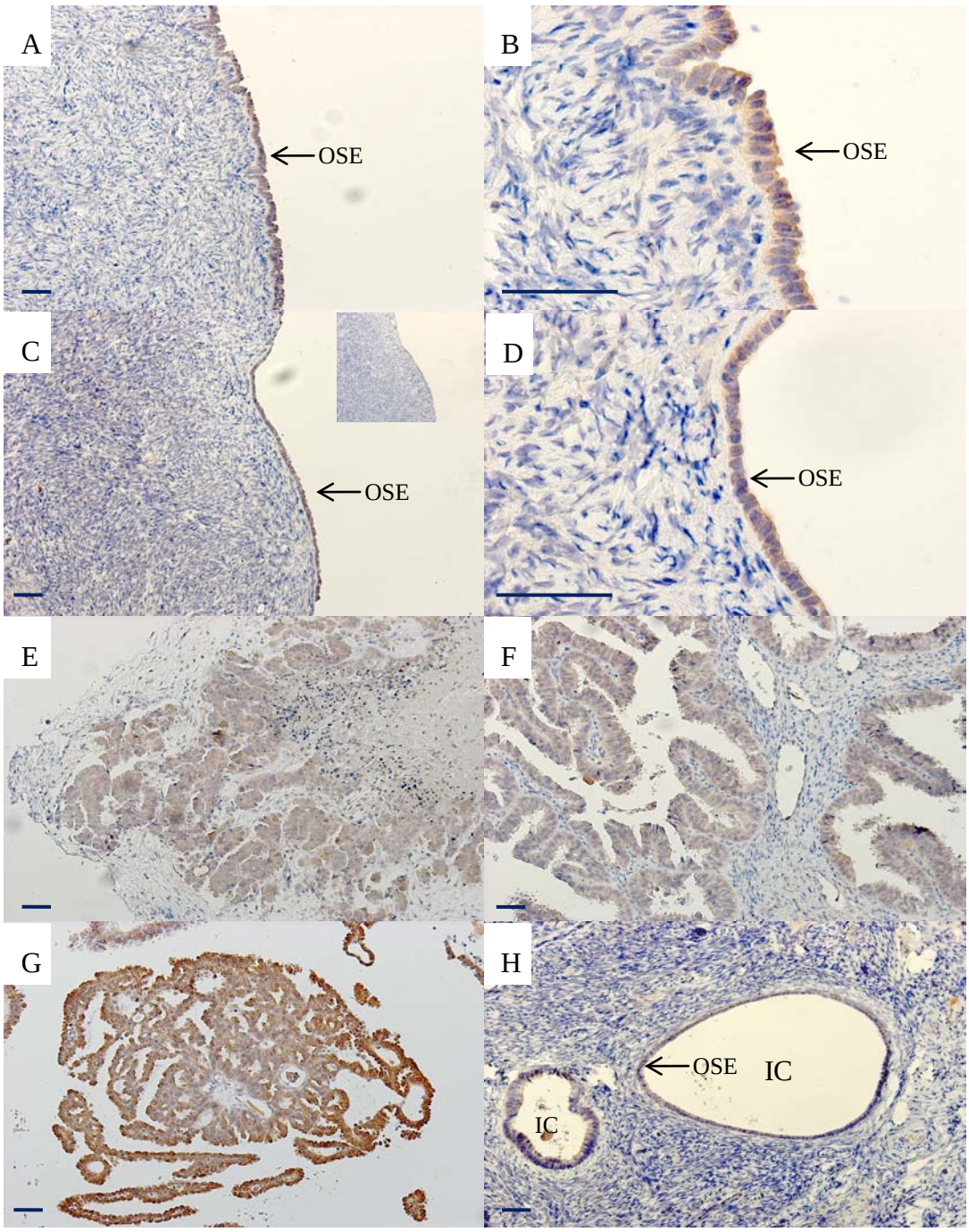

Figure 3.1: Immunostaining of STS in human pre-menopausal ovaries, post-menopausal ovaries and ovarian cancers. All the tissues were fixed and stained with STS antibody as described in Section 2.3. A, B) Pre-menopausal ovary with OSE; C, D) Post-menopausal ovary with OSE, insert shows negative control; E) Serous ovarian adenocarcinoma; F) Mixed differentiation ovarian carcinoma with mucinous, serous and endometrioid features; G) Serous and endometrioid ovarian adenocarcinoma; H) Pre-menopausal ovary with inclusion cysts. Scale bars 40µm.

3.3.2 Expression of EST protein in human ovary and ovarian cancer

EST protein was expressed in pre-menopausal ovary, post-menopausal ovary, inclusion cysts and ovarian cancer. Expression was mainly localised to OSE in normal ovary, epithelium of inclusion cysts and ovarian cancer cells in ovarian cancer, with relatively low expression seen in the endothelial cells and stromal cells in all the samples. Low expression of EST in granulosa and theca cells was observed in one pre-menopausal ovary (data not shown). There was no difference in expression patterns between pre-menopausal and post-menopausal ovaries. Intensity of expression did not differ among pre-menopausal OSE, post-menopausal OSE, OSE embedded in inclusion cysts and ovarian cancer cells. These data are shown in Figure 3.2.

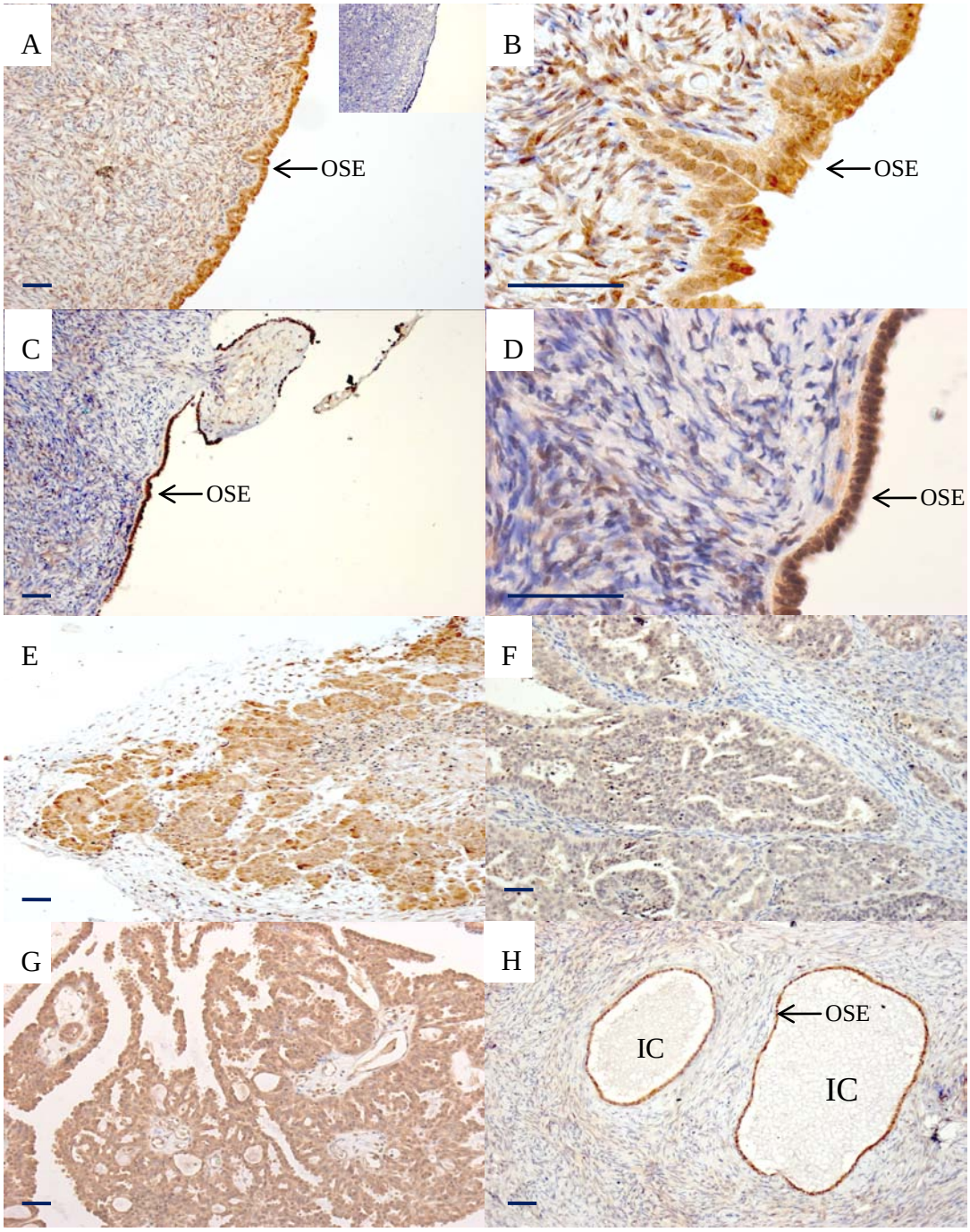

Figure 3.2: Immunostaining of EST in human pre-menopausal, post-menopausal ovaries and ovarian cancers. All the tissues were fixed and stained with EST antibody as described in Section 2.3. A, B) Pre-menopausal ovary with OSE, insert shows negative control; C, D) Post-menopausal ovary with OSE; E) Serous ovarian adenocarcinoma; F) Endometrioid ovarian adenocarcinoma; G) Serous and endometrioid ovarian adenocarcinoma; H) Pre-menopausal ovary with inclusion cysts. Scale bars 40µm.

3.3.3 Expression of 17βHSD5 protein in human ovary and ovarian cancer

17βHSD5 protein was expressed in pre-menopausal ovary, post-menopausal ovary, inclusion cysts and ovarian cancer. Expression was mainly localised to OSE in normal ovary, epithelium of inclusion cysts, ovarian cancer cells in ovarian cancer and endothelial cells in all the samples, with very low expression seen in stromal cells in some samples and in granulosa and theca cells of one pre-menopausal ovary (data not shown). There was no difference in expression patterns between pre-menopausal and post-menopausal ovaries. Intensity of expression did not differ among pre-menopausal OSE, post-menopausal OSE, OSE embedded in inclusion cysts and ovarian cancer cells. These data are shown in Figure 3.3.

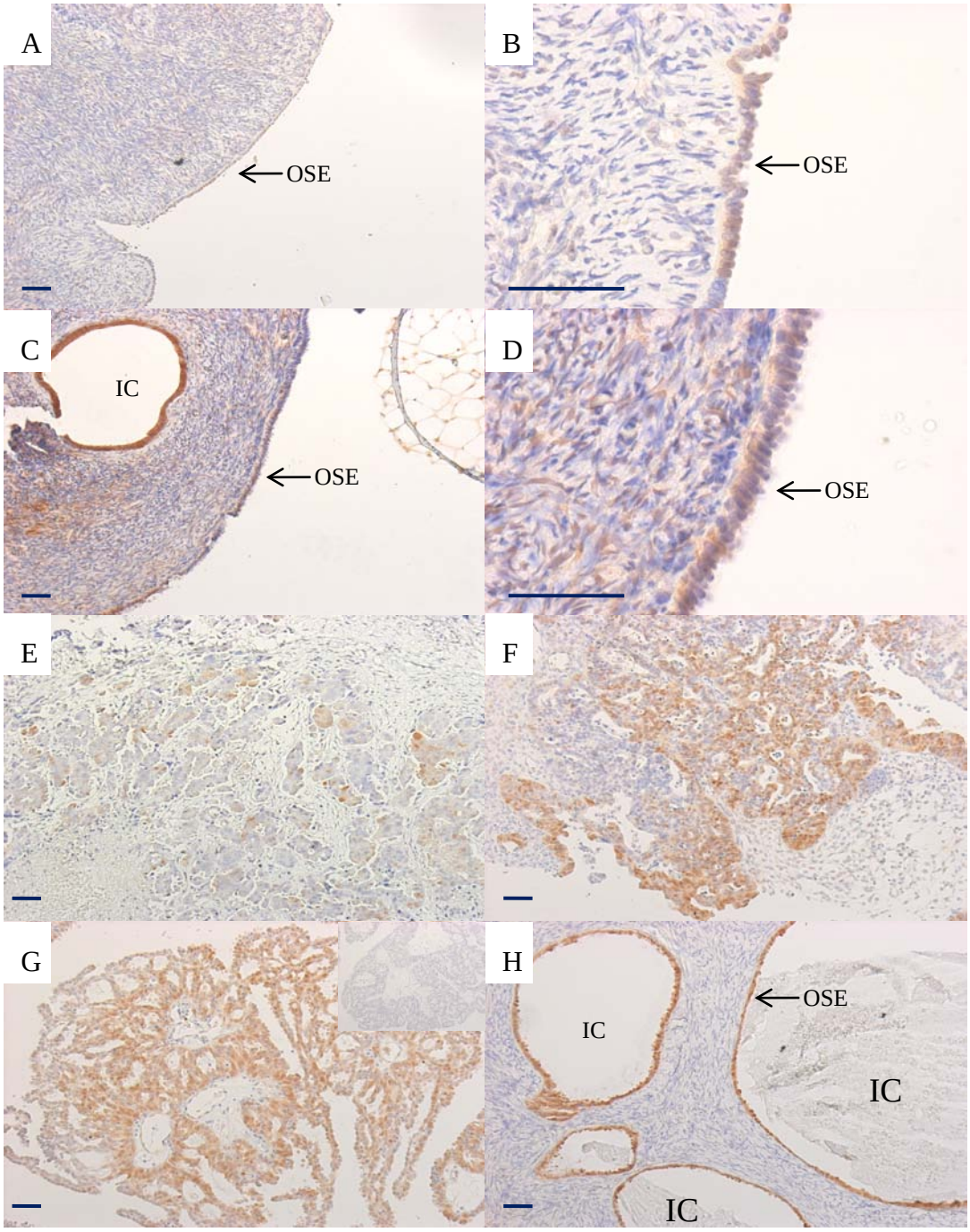

Figure 3.3: Immunostaining of 17βHSD5 in human pre-menopausal, post-menopausal ovaries and ovarian cancers. All the tissues were fixed and stained with 17βHSD5 antibody as described in Section 2.3. A, B) Pre-menopausal ovary with OSE; C, D) Post-menopausal ovary with OSE; E) Serous ovarian adenocarcinoma; F) Endometrioid ovarian adenocarcinoma; G) Serous and endometrioid ovarian adenocarcinoma, insert shows negative control; H) Pre-menopausal ovary with inclusion cysts. Scale bars 40µm.

3.3.4 Expression of 17βHSD2 protein in human ovary and ovarian cancer

17βHSD2 protein was expressed in pre-menopausal ovary, post-menopausal ovary, inclusion cysts and ovarian cancer. Expression was mainly localised to OSE in normal ovary, epithelium of inclusion cysts and ovarian cancer cells in ovarian cancer, with moderate expression in granulosa cells and low expression in theca cells of two pre-menopausal ovaries (data not shown). The expression seen in the endothelial and stromal cells in most of the samples was very low. There was no difference in expression patterns between pre-menopausal and post-menopausal ovaries. Intensity of expression did not differ between pre-menopausal OSE, post-menopausal OSE, OSE embedded in inclusion cysts and ovarian cancer cells. These data are shown in Figure 3.4.

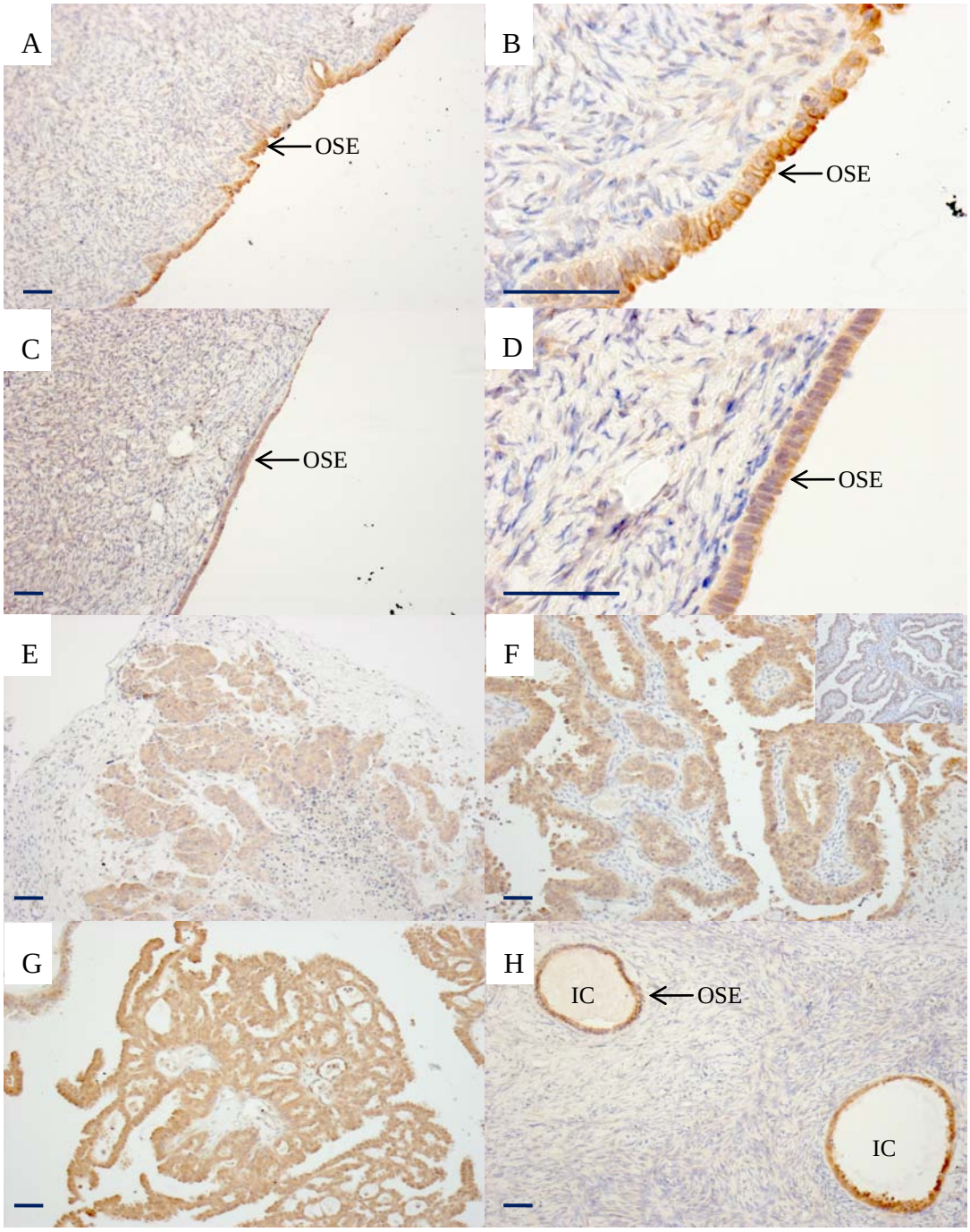

Figure 3.4: Immunostaining of 17βHSD2 in human pre-menopausal, post-menopausal ovaries and ovarian cancers. All the tissues were fixed and stained with 17βHSD2 antibody as described in Section 2.3. A, B) Pre-menopausal ovary with OSE; C, D) Post-menopausal ovary with OSE; E) Serous ovarian adenocarcinoma; F) Endometrioid ovarian adenocarcinoma, insert shows negative control; G) Serous and endometrioid ovarian adenocarcinoma; H) Pre-menopausal ovary with inclusion cysts. Scale bars 40µm.

3.3.5 Expression of aromatase protein in human ovary and ovarian cancer

IHC revealed no aromatase protein expressed in pre-menopausal ovary, post-menopausal ovary, inclusion cysts and ovarian cancer samples. Term-birth placenta showed intense aromatase expression in trophoablast cell. These data are shown in Figure 3.5.

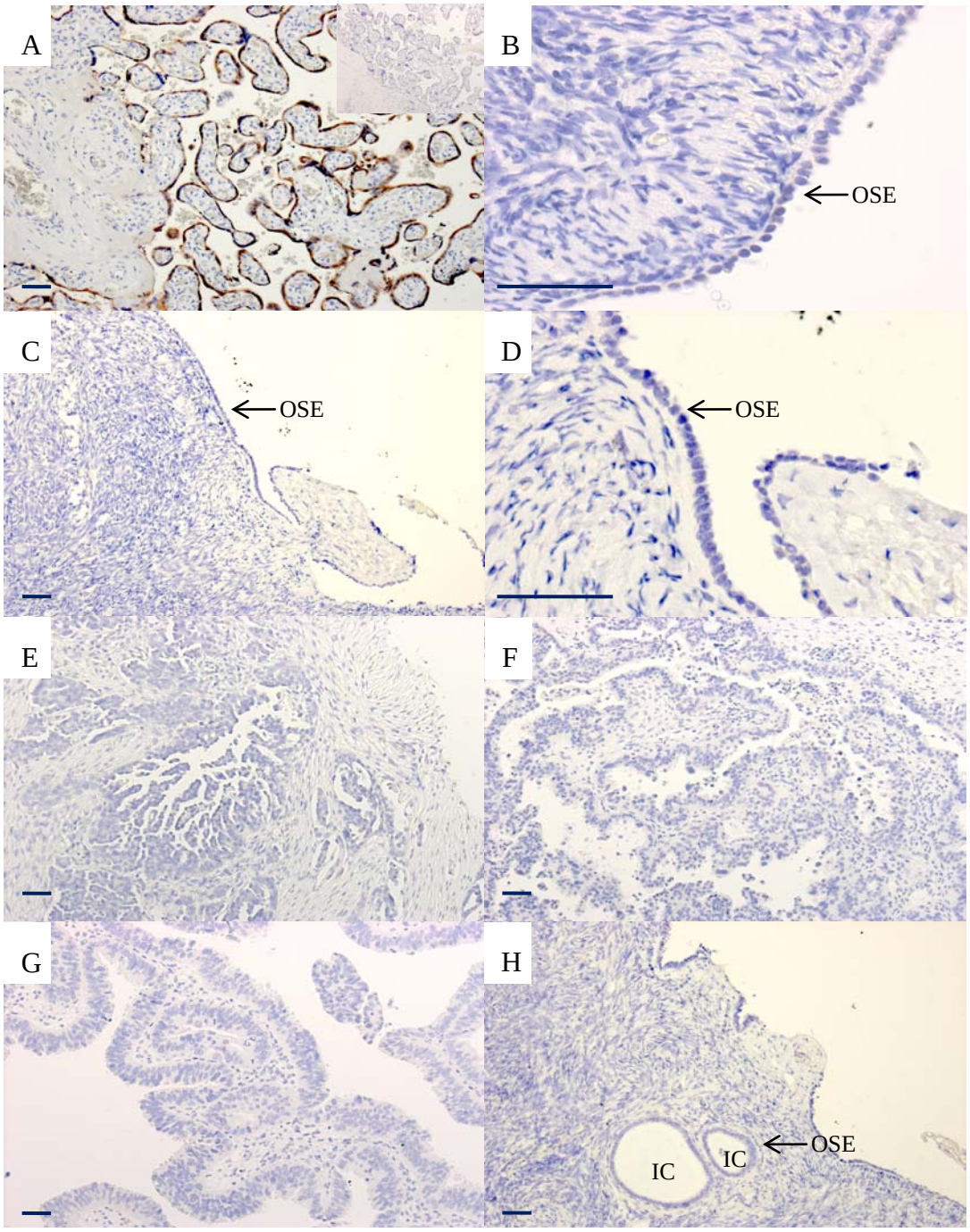

Figure 3.5: Immunostaining of aromatase in human placenta, pre-menopausal, post-menopausal ovaries and ovarian cancers. All the tissues were fixed and stained with aromatase antibody as described in Section 2.3. A) Placenta, insert shows negative control; B) Pre-menopausal ovary with OSE; C, D) Post-menopausal ovary with OSE; E) Serous ovarian adenocarcinoma; F) Endometrioid ovarian adenocarcinoma; G) Mixed differentiation ovarian carcinoma with mucinous, serous and endometrioid features; H) Pre-menopausal ovary with inclusion cysts. Scale bars 40µm.

3.4 Discussion

These studies show that STS, EST, 17βHSD2 and 17βHSD5 proteins are all expressed in pre- and post-menopausal OSE, epithelium lining inclusion cysts and ovarian cancer cells, although the levels of expression did not vary between these cells. Aromatase protein was undetectable in any of the samples, although the positive control sample showed intense expression. These data demonstrate for the first time the expression and localisation of EST protein in human ovary, ovarian cancer and inclusion cysts, and show for the first time expression of these oestrogen-producing enzymes in ovarian inclusion cysts.

STS is the enzyme responsible for the conversation of E_1S to E_1. In this study STS protein was present in pre- and post-menopausal OSE and OSE of inclusion cysts. This is the first report showing STS protein expression in OSE cells, and indicates the possibility of local production of E_1 through STS activity in OSE cells. Interestingly, there was also STS protein expression in OSE cells lining inclusion cysts. Since the microenviroment around inclusion cysts is vital for the transformation of the normal cells to cancer cells, E_2 biosynthesized locally may be a very important factor involved in the tumourigenic process, if STS is active in these OSE cells. The expression of STS protein in ovarian cancer tissues was consistent with previously published data. Okuda and co-workers showed STS immunostaining in 70% of ovarian clear cell adenocarcinoma (32/45), 33.3% of serous adenocarcinoma (6/18) and 50% of mucinous adenocarcinoma (4/8) (Okuda, Saito et al. 2001). All 6 of the ovarian cancer samples in this study, including 3 serous carcinoma samples, 2 endometrioid samples, 1 mixed differentiation carcinoma with mucinous, serous and endometrioid features and 1 mixed serous and endometrioid carcinoma, demonstrated positive STS staining. These results confirm the previous study (Okuda, Saito et al. 2001), showing that STS protein is widely expressed in different kinds of epithelial ovarian cancers. Chura and co-workers detected STS enzymatic activity in 36 of 37 advanced ovarian cancer specimens by TLC, further supporting the involvement of STS in local oestrogen production in EOC (Chura, Blomquist et al. 2009). The expression of STS protein in OSE cells and ovarian

cancer cells suggested its potential role in the biosynthesis of E_1 in these cells, however, its enzymatic activity need to be tested to confirm local E_1 formation.

EST, which opposes the action of STS, sulphonates E_1 to biologically inactive E_1S. Therefore EST is considered to be involved in the regulation of E_2 levels *in situ* together with STS. The local production of E_2 depends on the balance between activities of these two enzymes. EST protein was reported previously in the cytoplasm of invasive ductal breast carcinoma cells by IHC (Suzuki, Nakata et al. 2003). In addition, EST protein was found in prostate carcinoma cells (Nakamura, Suzuki et al. 2006) and in 45% of colon carcinomas (Sato, Suzuki et al. 2009). However, there are no previous reports of EST expression patterns in human ovary and ovarian cancer. In the present study a polyclonal antibody for mouse EST was used to localize EST protein. Because human and mouse EST are 77% homologous at the amino acid level, it was expected that this mouse antibody would also bind to the human EST, as demonstrated in a study showing that the mouse EST antibody was able to recognize human EST (Song, Qian et al. 1998). However, the antibody also cross-reacted with the expressed human phenol sulfotransferases (PST) which are responsible for sulpho-conjugating monoamine neurotransmitters and drugs or xenobiotic compounds such as phenols (Song, Qian et al. 1998). Nevertheless this was the only available antibody for the current study. EST protein was firstly reported in pre- and post-menopausal OSE cells, OSE cells lining inclusion cysts and ovarian cancer cells, suggesting EST may play an important role together with STS in regulating the local production of E_2 in OSE and ovarian cancer and then influence the development of ovarian cancer. Although there is no quantifiable difference of the EST expression between different OSE cells and ovarian cancer cells, given the limitation of the analysis of IHC and the specificity of the antibody, quantitative analysis of STS and EST mRNA and measurement of enzymatic activities in different cell types was performed to compare the local formation of E_2 via the STS pathway (see Chapter 4).

A previous study reported aromatase immunostaining in OSE cells but did not clarify the percentage of positive staining among all the tested samples, and the multilayered OSE described is unlike the OSE in the present study, being more representative of the granulosa cell layer in a large antral follicle (Cunat, Rabenoelina et al. 2005). Okubo measured aromatase activity in a human OSE cell line (HOSE17) but did not test it on primary OSE cells (Okubo, Mok et al. 2000). Aromatase expression in ovarian cancer has been extensively studied. For example, active aromatase enzymatic activity was found in three epithelial ovarian adenocarcinoma cell lines suggesting that at least some adenocarcinoma possess sufficient aromatase activity to convert ovarian stromal androgen to oestrogen (Thompson, Adelson et al. 1988). In addition, Noguchi determined aromatase activity in 81% of 35 ovarian tumours (Noguchi, Kitawaki et al. 1993) and Kitawaki detected aromatase activity in 68% (17/25) of the malignant ovarian tumours and 82% (22/27) of benign tumours (Kitawaki, Noguchi et al. 1996). Interestingly, Kaga found aromatase expression in stromal cells surrounding the carcinomatous nests of 79.5% ovarian carcinomas but not in cancer cells (Kaga, Sasano et al. 1996). In Cunat's study, aromatase mRNA expression was detected in 49% of ovarian cancer specimens and BG1, PEO-4 and PEO-14, but not in SKOV-3 and OVCAR-3 cell lines. Aromatase activity was also confirmed in BG1 and PEO-14 cell lines (Cunat, Rabenoelina et al. 2005). In contrast, Imai did not detect aromatase mRNA expression or enzyme activity in three mucinous and one serous cystadenocarcinoma specimens (Imai, Ohno et al. 1994). It therefore remains unclear whether both EOC and OSE produce active oestrogen through aromatase activity. There are two main reasons why our study may have failed to detect aromatase activity in the ovarian and EOC samples. Firstly, we used only 6 post- and 3 pre-menopausal ovaries, and 7 ovarian cancer samples. Given the limited numbers it was possible there was no aromatase expression in these samples, since previous studies have demonstrated aromatase expression exists in only a proportion of ovarian cancers. Secondly, aromatase protein in the ovary and ovarian cancer might be much lower than in placenta, which could explain why the expression appeared negative in OSE and EOC cells while positive in trophoblast cells despite the same experimental primary antibody concentration. The positive staining in trophoblast cells was the same expression pattern reported in previous

paper (Colette, Lousse et al. 2009). Use of a higher concentration of antibody might have revealed low expression of aromatase in OSE and EOC cells.

17βHSD2 possesses oxidative activity, inactiving E_2 by converting it into E_1 whereas 17βHSD5 acts as reductase converting E_1 into E_2. Therefore the interconversion of E_1 and E_2 is a bi-directional equilibrium reaction rather than unidirectional metabolism, and the balance of the expression and activity of these two enzymes is important for determining the local production of E_2. There are a few reports showing their presence in ovarian cancer, but not in OSE cells. Vierikko detected 17βHSD activities in a series of benign and malignant epithelial tumours (Vierikko, Kauppila et al. 1983). Blomquist demonstrated both 17βHSD2 and 17βHSD5 activities in seven mucinous, three serous and two endometrioid tumours (Blomquist, Bonenfant et al. 2002) and Provost detected 17βHSD2 and17βHSD5 activities in one ovarian cancer cell line, OV-90, which was derived from patients unexposed to radiation or chemotherapy (Provost, Lima et al. 2010). Our results are in agreement with the above studies confirming the expression of both 17βHSD2 and 17βHSD5 proteins in ovarian cancer. Furthermore, we show for the first time the expression of these two proteins in pre- and post-menopausal OSE cells as well as epithelial cells lining the inclusion cysts, suggesting a potential role of these two enzymes in maintaining the balance between E_1 and E_2 in these cells.

One aspect of interest that arose from our immunohistochemical studies was the weak positive expression of STS, EST, 17βHSD2 and 17βHSD5 proteins in stromal cells in some of the ovary and ovarian cancer samples. This provides a clue that the production of E_2 might occur not only in OSE or ovarian cancer cells but also in some stromal cells. Therefore the E_2 formed by OSE and cancer cells might act in an intracrine manner and E_2 produced by stromal cells influence the cancer cells as a paracrine factor. Thus stromal cells might interact together with the epithelium and cancer cells during the development of ovarian cancer. This is supported by a clinical case reported by Tokunaga, in which an ovarian epithelial carcinoma patient had a higher serum E_2 due to intratumoural oestrogen production by both cancer and

stromal cells, with enzymes required for the production of oestrogen present in both cancer and stromal cells (Tokunaga, Akahira et al. 2007).

One weakness of the study in this chapter is the limited number of the samples. The results would be more convincing and we would be able to compare the differences in the target gene expression between different EOC subtypes if we had been able to include more samples for each type of EOC. Although IHC is very suitable for localizing the cellular origin of protein it does not permit accurate quantification. In Chapter 4, Taqman quantitative RT-PCR was used to test the mRNA levels of these genes.

In summary, STS, EST, 17βHSD2 and 17βHSD5 proteins are expressed at different levels in pre-menopausal OSE, post-menopausal OSE, epithelium lining inclusion cysts and ovarian cancer cells and there is no clear difference in expression of these genes between different types of cells. Aromatase protein was not detected in these cells. Thus we can conclude that there might be the local production of E_2 by these active genes in the above cells. Table 3.1 shows a summary of the data presented in this chapter.

	Pre-menopausal ovary			Post-menopausal ovary			EOC			Inclusion cysts
	OSE	**Stroma**	**Endothelium**	**OSE**	**Stroma**	**Endothelium**	**Cancer cells**	**Stroma**	**Endothelium**	**OSE**
STS	++	+	+	++	+	+	++	+	+	++
EST	+++	+	++	+++	+	++	+++	+	++	+++
Aromatase	-	-	-	-	-	-	-	-	-	-
17βHSD2	++	+	+	++	+	+	++	+	+	++
17βHSD5	++	+	++	++	+	++	++	+	++	++

Table 3.1: Summary of relative protein expression of different oestrogen metabolizing genes.

- no staining, + mild staining, ++ moderate staining, +++ strong staining

Chapter 4

Expression of genes encoding the metabolism and action of oestrogen in pre-menopausal OSE, EOC and ovarian cancer cell lines

4.1 Introduction

Having established the protein expression of oestrogen metabolizing genes in OSE, EOC and OSE lining the inclusion cysts in Chapter 3, and demonstrating the possibility of local oestrogen production in these cells, it was then important to investigate differences in relative mRNA levels between these cells and also test the enzyme activities to investigate if these cells have differential capacities to produce E_2.

The expression of STS and 17βHSD5 proteins in the OSE and EOC confirmed by IHC in Chapter 3 indicates the possible production of E_2 in these cells from E_1S via the STS pathway, rather than from androgens via the aromatase pathway. However, E_1S carries a net negative charge at physiological pH levels and, as such, its transfer across cell membranes is carrier mediated. Steroid sulphates have been identified as substrates for distinct members of two organic anion carrier gene families: OATP super family and OAT family. There are many subtypes of OATPs and OATs but studies of breast cancer and placenta have demonstrated OATP-B, OATP-D, OATP-E and OAT4 are the main candidate transporters of E_1S (Pizzagalli, Varga et al. 2003; Wlcek, Svoboda et al. 2008). Because the transport processes operative in the tissue will govern the cellular entry of conjugated steroid, these transporters may be determinants of downstream oestrogen exposure in target cells. Therefore, a further aim of this chapter is to identify and characterize the organic anion uptake system for E_1S, namely to compare OATP-B, OATP-D, OATP-E and OAT4 mRNA expression in OSE and EOC.

Since most of ovarian cancers occur after menopause and they are thought to derive from the OSE cells trapped as an epithelium in inclusion cysts, it would be ideal to compare gene expression in pre-menopausal, post-menopausal and inclusion cyst OSE. However in this chapter it was only possible to study pre-menopausal OSE cells because there are very few patients undergoing operations due to benign diseases after menopause, which means very limited access to post-menopausal OSE. Moreover, it is extremely difficult to obtain OSE cells lining inclusion cysts due to technical limitations and limited cell numbers. Due to the limited availability of

primary cancer tissue, we also chose three cancer cell lines, PEO-1, SKOV-3 and PEO-14. The first two are both from poorly-differentiated serous ovarian cancer patients and have high expression of oestrogen receptors while the latter is from a well-differentiated serous ovarian cancer patient and has low expression of oestrogen receptors.

In summary, the objective of this chapter is to compare mRNA levels of different oestrogen metabolism and action genes between pre-menopausal OSE and cancer cells and evaluate the activity of the oestrogen metabolizing enzymes in normal and cancer cells.

4.2 Materials and methods

4.2.1 OSE, EOC cells and ovarian cancer cell lines

Pre-menopausal OSE and EOC cells were collected and cultured as described in Sections 2.1 and 2.2. The basic clinical profiles of patients involved in the respective assays are presented Table 4.1 below. Information on the ovarian cancer cell lines was listed previously in Section 2.2.1.

Patient No	**Code**	**LREC No**	**Age**	**Reason for surgery**	**Surgery**	**Experimental Study**
1	6321	04/S1103/36	26	Pelvic pain	DiagLapar	qRT-PCR
2	6800	04/S1103/36	50	Fibroids	TAHBSO	qRT-PCR
3	7527	04/S1103/36	38	Prophylactic	LapSter	qRT-PCR
4	7538	04/S1103/36	21	Pelvic pain	DiagLapar	qRT-PCR
5	7543	04/S1103/36	26	Prophylactic	LapSter	qRT-PCR
6	7548	04/S1103/36	31	Prophylactic	LapSter	qRT-PCR
7	7549	04/S1103/36	29	Prophylactic	LapSter	qRT-PCR
8	7565	04/S1103/36	32	Pelvic pain	DiagLapar	qRT-PCR
9	7566	04/S1103/36	41	Pelvic pain	DiagLapar	qRT-PCR
10	7606	04/S1103/36	36	Prophylactic	LapSter	qRT-PCR
11	7613	04/S1103/36	48	Fibroids	TAH	qRT-PCR
12	7623	04/S1103/36	49	Fibroids	TAHBSO	qRT-PCR
13	6394	04/S1103/36	29	HMB	DiagLapar	qRT-PCR
14	6829	04/S1103/36	45	Fibroids	TAHBSO	qRT-PCR
15	6825	04/S1103/36	43	Fibroids	TAHBSO	qRT-PCR
16	CA11	04/S1103/44	N/A	N/A	N/A	qRT-PCR
17	CA9B	04/S1103/44	N/A	N/A	N/A	qRT-PCR
18	44021	04/S1103/44	55	EOC	N/A	qRT-PCR
19	44028	04/S1103/44	59	EOC	N/A	qRT-PCR
20	44029	04/S1103/44	67	EOC	N/A	qRT-PCR

Patient No	Code	LREC No	Age	Reason for surgery	Surgery	Experimental Study
21	44035	04/S1103/44	66	EOC	N/A	qRT-PCR
22	3028	04/S1103/36	29	Pelvic pain	DiagLapar	TLC
23	5617	04/S1103/36	27	Prophylactic	LapSter	TLC
24	6316	04/S1103/36	43	HMB and pelvic pain	TAHBSO	TLC
25	6318	04/S1103/36	41	Hydrosalpinx	Romoval of Hydrosalpinx	TLC
3	7527	04/S1103/36	38	Prophylactic	LapSter	TLC
4	7538	04/S1103/36	21	Pelvic pain	DiagLapar	TLC
26	7541	04/S1103/36	33	Prophylactic	LapSter	TLC
27	7544	04/S1103/36	34	Prophylactic	LapSter	TLC
28	7546	04/S1103/36	19	Left ovarian cyst	DiagLapar	TLC
29	7550	04/S1103/36	31	Prophylactic	LapSter	TLC

Table 4.1: clinical profile of patients used for qRT-PCR and TLC.

DiagLapar: diagnostic laparoscopy; LapSter: laparoscopic sterilization; HMB: heavy menstruation bleeding; LREC: Lothian Research Ethical Committee; N/A: not available; TAH: total abdominal hysterectomy; TAHBSO: total abdominal hysterectomy and bilateral salpingo-oophorectomy; TLC: thin layer chromatography.

4.2.2 RNA extraction and reverse transcription

RNA was extracted using methods described in Section 2.4.1 and RT-PCR was performed using standard conditions as described in Section 2.4.2. Equal concentrations (200ng) of DNase-treated RNA extracted from OSE and EOC samples of 17 and 4 patients respectively were reverse transcribed and measured for oestrogen metabolic pathway transcript levels.

4.2.3 Taqman qRT-PCR

QRT-PCR was performed using an ABI 7900 sequence detection system and analysed as described in Section 2.4.3. The probes and primers used were commercially available pre-validated from Applied Biosystems Assay on Demand or designed in house. The information on the primers and probes is shown in Table 2.3.

4.2.4 Enzyme activity assay

The activities of the target enzymes were tested using the method described in Section 2.5.

4.2.5 Statistical analysis

Statistical analysis was performed using GraphPad Prism 5. QRT-PCR data were analyzed by Mann Whitney test. Data from enzyme activity assay were analyzed by one-way analysis of variance (ANOVA) with Tukey post-hoc test. Data for the Glusulase (Glu) treatment were analyzed by paired T tests. Statistical difference was assigned at $P<0.05$.

4.3 Results

4.3.1 Expression of oestrogen metabolic pathway genes in pre-menopausal OSE and EOC

STS mRNA was expressed in all the OSE and EOC samples although there was no significant difference between OSE and EOC samples (Fig. 4.1, A). EST mRNA was expressed at detectable levels in 16 of 17 OSE and 2 of 4 EOC samples and the expression of EST in EOC was significantly lower than in OSE samples (Fig. 4.1, B; $P<0.05$). Aromatase mRNA was detectable in 11 of 17 OSE and all the EOC samples. Although the median OSE mRNA was lower than EOC mRNA there was no statistically significant difference. For aromatase, placenta mRNA was used as the standard and its level was more than 10^3 times higher than the expression in OSE and EOC samples (Fig. 4.1, C). 17βHSD1 mRNA was expressed in all the OSE and 2 of 4 EOC samples and there was no significant difference between OSE and EOC samples (Fig. 4.2, A). 17βHSD2 mRNA was expressed in all the OSE and 3 of 4 EOC samples and there was no significant difference between OSE and EOC samples (Fig. 4.2, B). 17βHSD5 mRNA was expressed in all the OSE and EOC samples and there was no significant difference between OSE and EOC samples (Fig. 4.2, C). ERα mRNA was detectable in all the OSE and EOC samples and there was no significant difference between OSE and EOC samples. There was one EOC sample with significantly high ERα mRNA, about 50 times higher than the other 3 EOC samples (Fig. 4.3, A). ERβ mRNA was detectable in less than half of the OSE samples (8 of 17) and 3 of 4 EOC samples and there was no significant difference between OSE and EOC samples (Fig. 4.3, B). OATP-B mRNA was detectable in 15 of 17 OSE and all the EOC samples. Its expression was significantly higher in EOC than in OSE cells (Fig. 4.4, A, $P<0.05$). OATP-D and OATP-E were expressed in all the OSE and EOC samples and there was no significant difference between OSE and EOC samples (Fig. 4.4, B and C). OAT4 mRNA expression was much lower than the placenta standard and was expressed in 6 of 17 OSE and 1 of 4 EOC samples and there was no significant difference between OSE and EOC samples (Fig. 4.4, D).

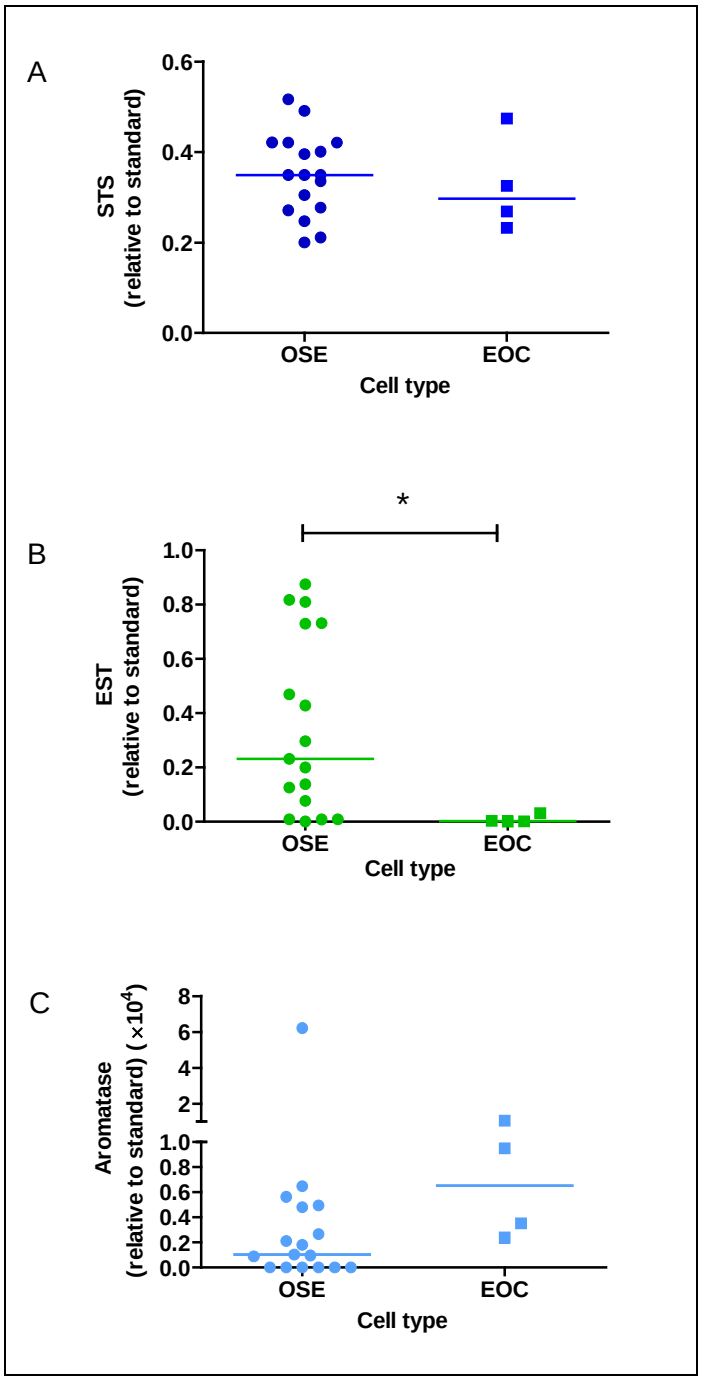

Figure 4.1: Measurement of STS (A), EST (B) and aromatase (C) mRNA expression in OSE (n=17) and EOC (n=4) by Taqman qRT-PCR. The standards were placenta (A, C) and proliferative endometrium (B). Horizontal bars indicate median value. (*=P<0.05)

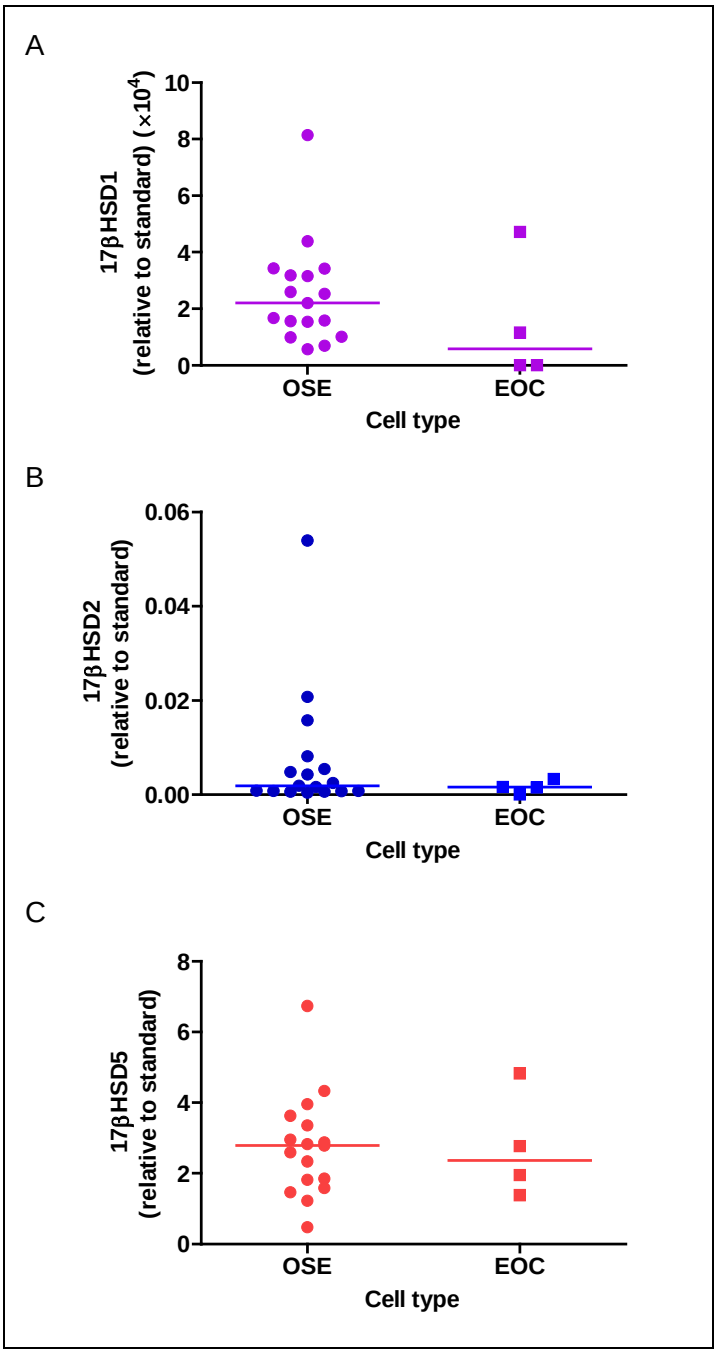

Figure 4.2: Measurement of 17βHSD1 (A), 17βHSD2 (B) and 17βHSD5 (C) mRNA expression in OSE (n=17) and EOC (n=4) by Taqman qRT-PCR. The standards were placenta (A, C) and proliferative endometrium (B). Horizontal bars indicate median value.

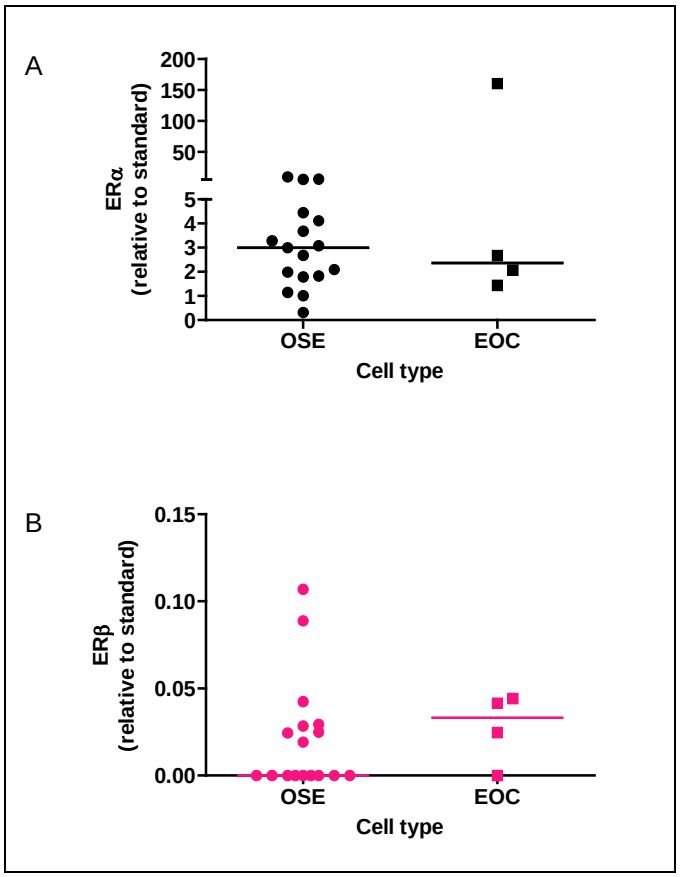

Figure 4.3: Measurement of ERα (A) and ERβ (B) mRNA expression in OSE (n=17) and EOC (n=4) by Taqman qRT-PCR. The standards were placenta. Horizontal bars indicate median value.

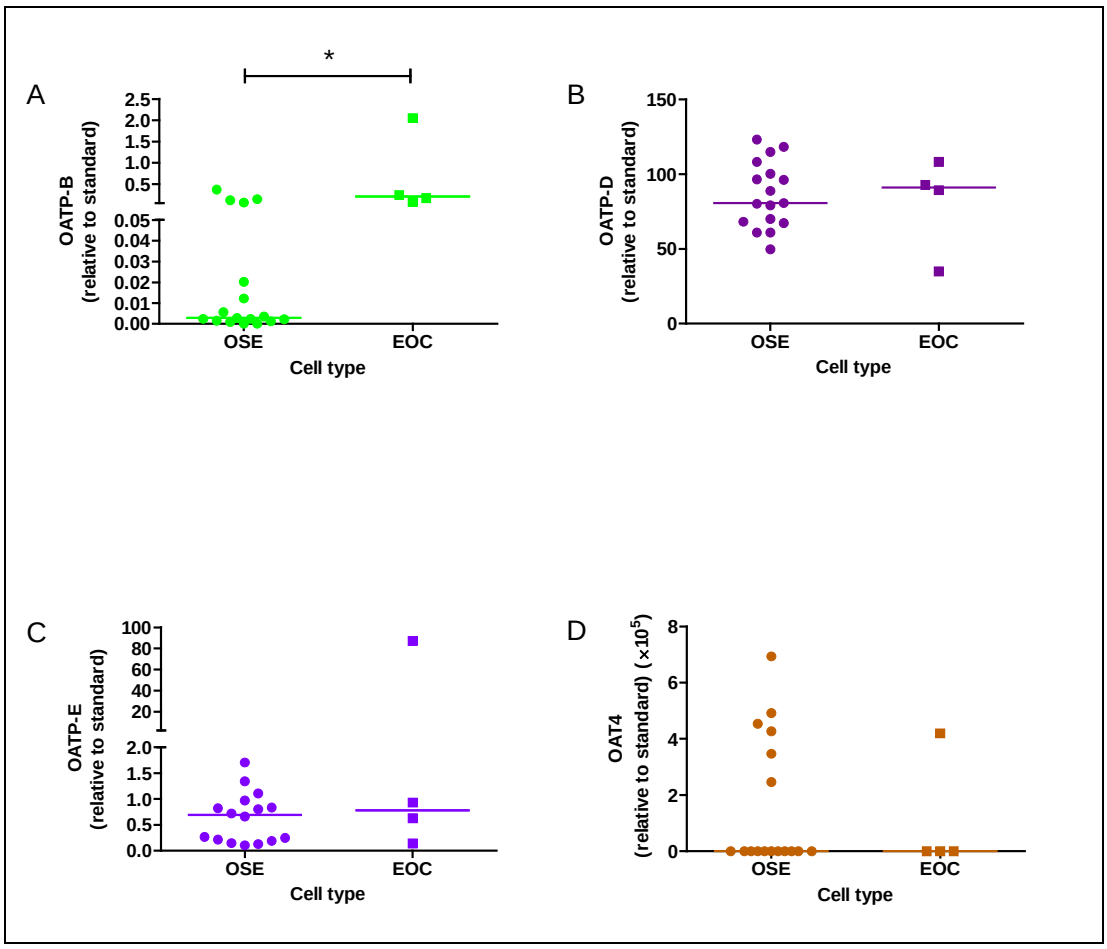

Figure 4.4: Measurement of OATP-B (A), OATP-D (B), OATP-E (C) and OAT4 (D) mRNA expression in OSE (n=17; for OATP-E, n=16) and EOC (n=4) by Taqman qRT-PCR. The standards were placenta. Horizontal bars indicate median value. (*=P<0.05)

4.3.2 Expression of oestrogen metabolic pathway genes in pre-menopausal OSE, EOC and ovarian cancer cell lines

To complement and extend the data on the limited primary ovarian cancer cells described above (Section 4.3.1), comparisons were then made between primary cells and PEO-14, SKOV-3 and PEO-1 ovarian cancer cell lines. Gene expression levels are presented in Figures 4.5, 4.6 and 4.7. Due to the mix of primary and cell line data, statistical comparisons of gene expression were not made. STS mRNA was expressed in OSE, EOC, PEO-14, SKOV-3 and PEO-1 cells and its expression was relatively lower in PEO-14 and SKOV-3 cells (Fig. 4.5, A). EST mRNA was highest in OSE, about 10-fold lower in EOC and PEO-14 and undetectable in SKOV-3 and PEO-1 cells (Fig. 4.5, B). For aromatase, placenta mRNA was used as the standard and its level was more than 10^3 times higher than the expression in OSE, EOC and PEO-14 cells. Aromatase mRNA was undetectable in SKOV-3 and PEO-1 cells (Fig. 4.5, C). 17βHSD1 mRNA was expressed in all the tested cells except PEO-1, and the level was higher in SKOV-3 cells than the other cell types (Fig. 4.6, A). 17βHSD2 mRNA was expressed in OSE, EOC and PEO-1 cells but the expression in all these cells was much lower than the expression in placenta standard. There was no 17βHSD2 mRNA expression detected in PEO-14 and SKOV-3 cells (Fig. 4.6, B). 17βHSD5 mRNA was expressed in all the cell types and there was much higher expression in SKOV-3 cells (about 100-fold higher than OSE) and relatively higher expression in PEO-14 cells (about 40-fold higher than OSE) (Fig. 4.6, C). ERα mRNA was expressed in OSE, EOC and PEO-14 cells at low levels while the expression in SKOV-3 and PEO-1 was more than 100-fold higher than the mean expression in OSE cells (Fig. 4.7, A). Compared to ERα there was little variation in the level of ERβ, although the latter was relatively higher in PEO-14 cells compared with the other cells (Fig. 4.7, B).

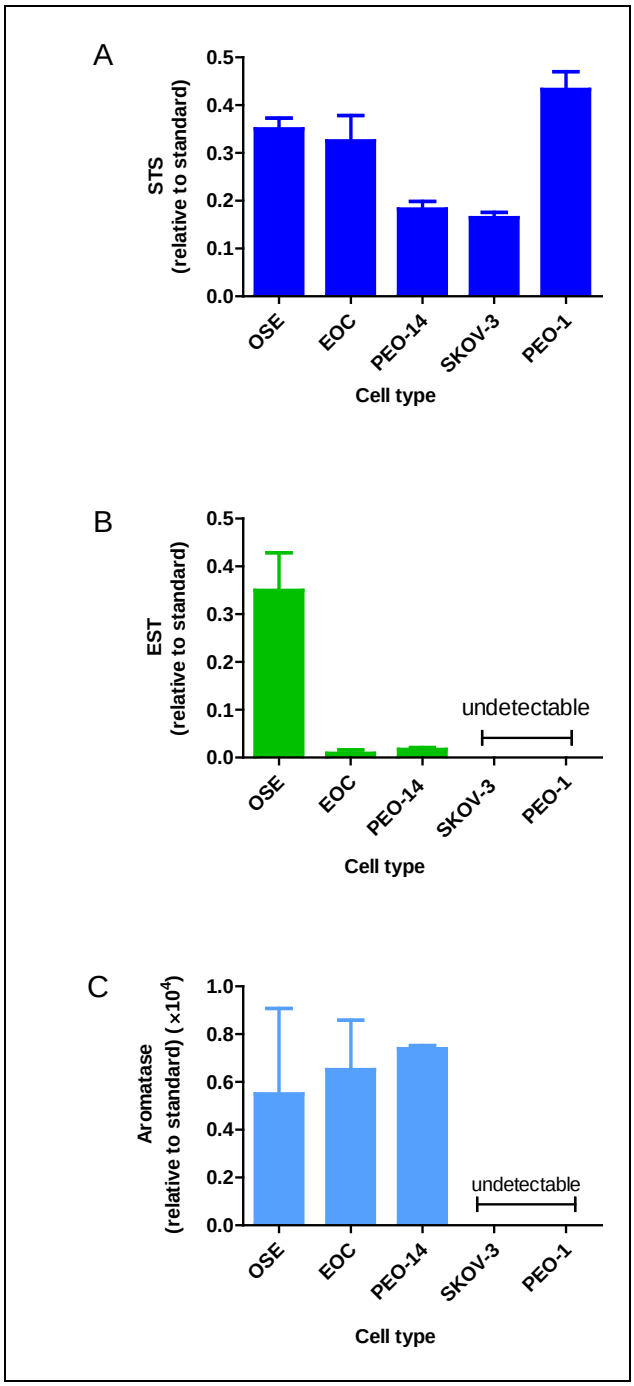

Figure 4.5: Measurement of STS (A), EST (B) and aromatase (C) mRNA expression in OSE, EOC, PEO-14, SKOV-3 and PEO-1 cells by Taqman qRT-PCR. The standards were placenta (A, C) and proliferative endometrium (B). Bars indicate Mean±standard error of the mean (SEM). No statistical comparisons were applied. (OSE, n＝17; EOC, n=4; cell lines, n=3)

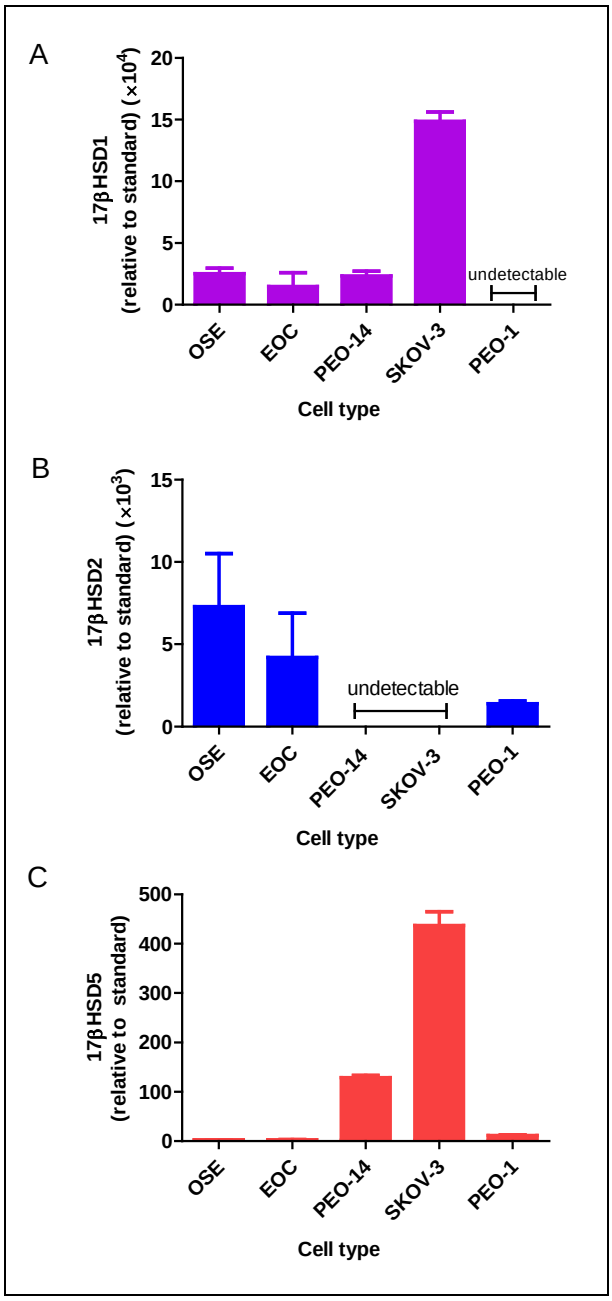

Figure 4.6: Measurement of 17βHSD1 (A), 17βHSD2 (B) and 17βHSD5 (C) mRNA expression in OSE, EOC, PEO-14, SKOV-3 and PEO-1 cells by Taqman qRT-PCR. The standards were placenta (A, C) and proliferative endometrium (B). Bars indicate Mean±SEM. No statistical comparisons were applied. (OSE, n=17; EOC, n=4; cell lines, n=3)

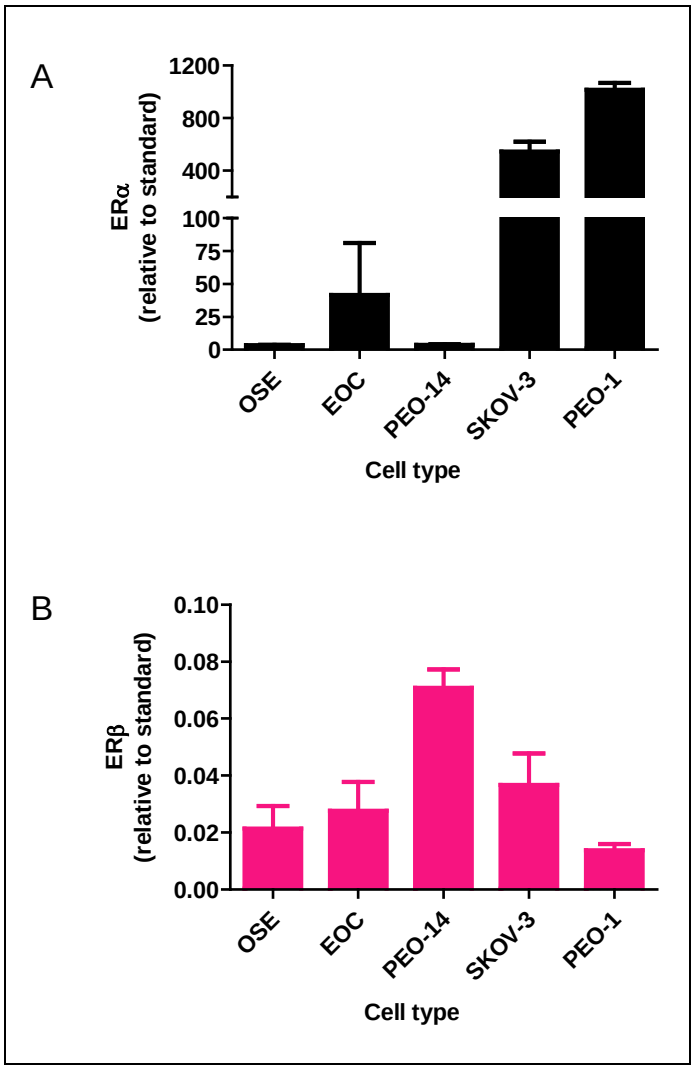

Figure 4.7: Measurement of ERα (A) and ERβ (B) mRNA expression in OSE, EOC, PEO-14, SKOV-3 and PEO-1 cells by Taqman qRT-PCR. The standards were placenta. Bars indicate Mean±SEM. No statistical comparisons were applied. (OSE, n=11; EOC, n=4; cell lines, n=3)

4.3.3 Oestrogen metabolizing enzyme activities in pre-menopausal OSE cells and ovarian cancer cell lines

4.3.3.1 Metabolism of [^{3}H]-E_1S in pre-menopausal OSE, SKOV-3 and PEO-1 cells

Oestrogen metabolizing enzyme activities in pre-menopausal OSE, SKOV-3 and PEO-1 cells were measured using a radiometric substrate conversion assay that involved measurement of [^{3}H]-E_1 and [^{3}H]-E_2 after treatment of the cells with [^{3}H]-E_1S as a substrate for 72h and separation according to their polarity/mobility on TLC. 500,000 cells were plated and serum-depleted for 24h before investigating the enzyme activities *in vitro*. In OSE cells there was no [^{3}H]-E_1 or [^{3}H]-E_2 converted from [^{3}H]-E_1S detected (Fig. 4.8, A). In contrast, SKOV-3 and PEO-1 cells converted [^{3}H]-E_1S to [^{3}H]-E_1 and further to [^{3}H]-E_2 (Fig. 4.8, B and C). When the radiographs were quantified as described in Section 2.5, the conversion rate of E_1S to E_1 or E_2 was significantly different between OSE and SKOV-3 or PEO-1 cells (Fig. 4.8, D and E, *=$P<0.05$, ***=$P<0.001$).

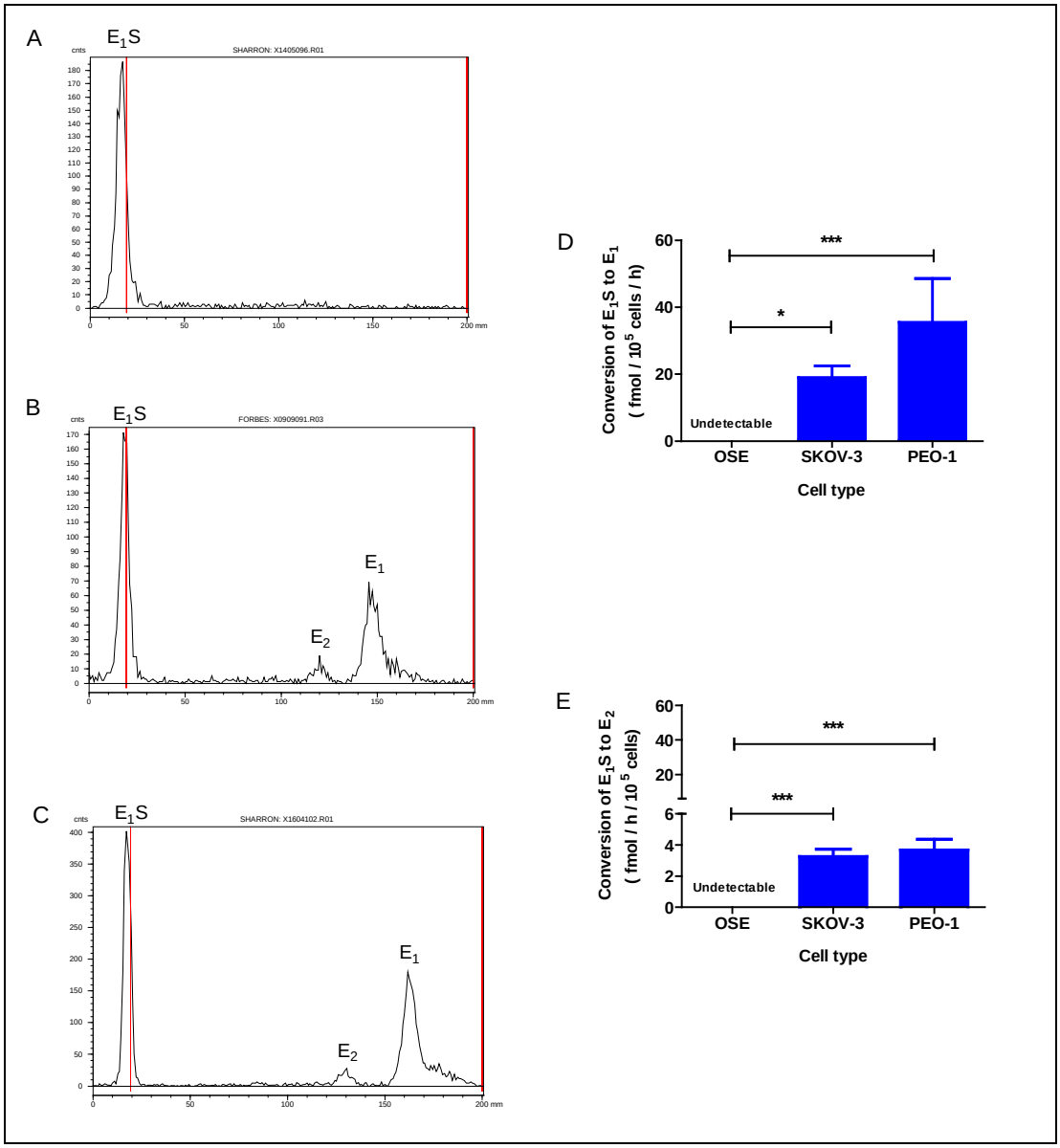

Figure 4.8: Radiochromatograph analysis of oestrogen pre-receptor metabolites in OSE (A, n=10), SKOV-3 (B, n=3) and PEO-1 (C, n=3) cells. Cells were cultured for 72h with [^{3}H]-E_1S and tritium labeled metabolites were detected by TLC and quantified by radioactivity scanner. Representative graphs indicate readout from TLC plates where y-axis shows counts of radioactivity and x-axis demonstrates the distance along the plate. Histograms show conversion rate of E_1S to E_1 (D) and E_1S to E_2 (E) calculated as described in Section 2.5. Bars indicate Mean±SEM. (*=P<0.05, ***=P<0.001)

4.3.3.2 Metabolism of [^{3}H]-E_1 in pre-menopausal OSE, SKOV-3 and PEO-1 cells

4.3.3.2.1 Metabolism of [^{3}H]-E_1

To investigate oestrogen metabolism enzyme activities in pre-menopausal OSE, SKOV-3 and PEO-1 cells further, and to determine whether E_1 is preferentially metabolized to E_1S or E_2, [^{3}H]-E_1 instead of [^{3}H]-E_1S was added as a substrate to treat the cells for 72h and radioactive metabolites after treatment were separated by TLC and measured by radioactivity scanner. 500,000 cells were plated and serum-depleted for 24h before investigating the enzymes activity *in vitro*. OSE cells converted most of [^{3}H]-E_1 back to the original position of [^{3}H]-E_1S on the chromatograph (Fig. 4.9, A) and this was confirmed by the glusulase experiment as described in Section 4.3.3.2.2. In contrast, SKOV-3 and PEO-1 cells converted part of [^{3}H]-E_1 to [^{3}H]-E_2 and a little to original position of [^{3}H]-E_1S (Fig. 4.9, B and C). When the radiographs were quantified as described in Section 2.5, there was a statistically significant difference in conversion rate of E_1 to E_1S or E_2 between OSE and SKOV-3 or PEO-1 cells (Fig. 4.9, D and E, *=$P<0.05$, ***=$P<0.001$).

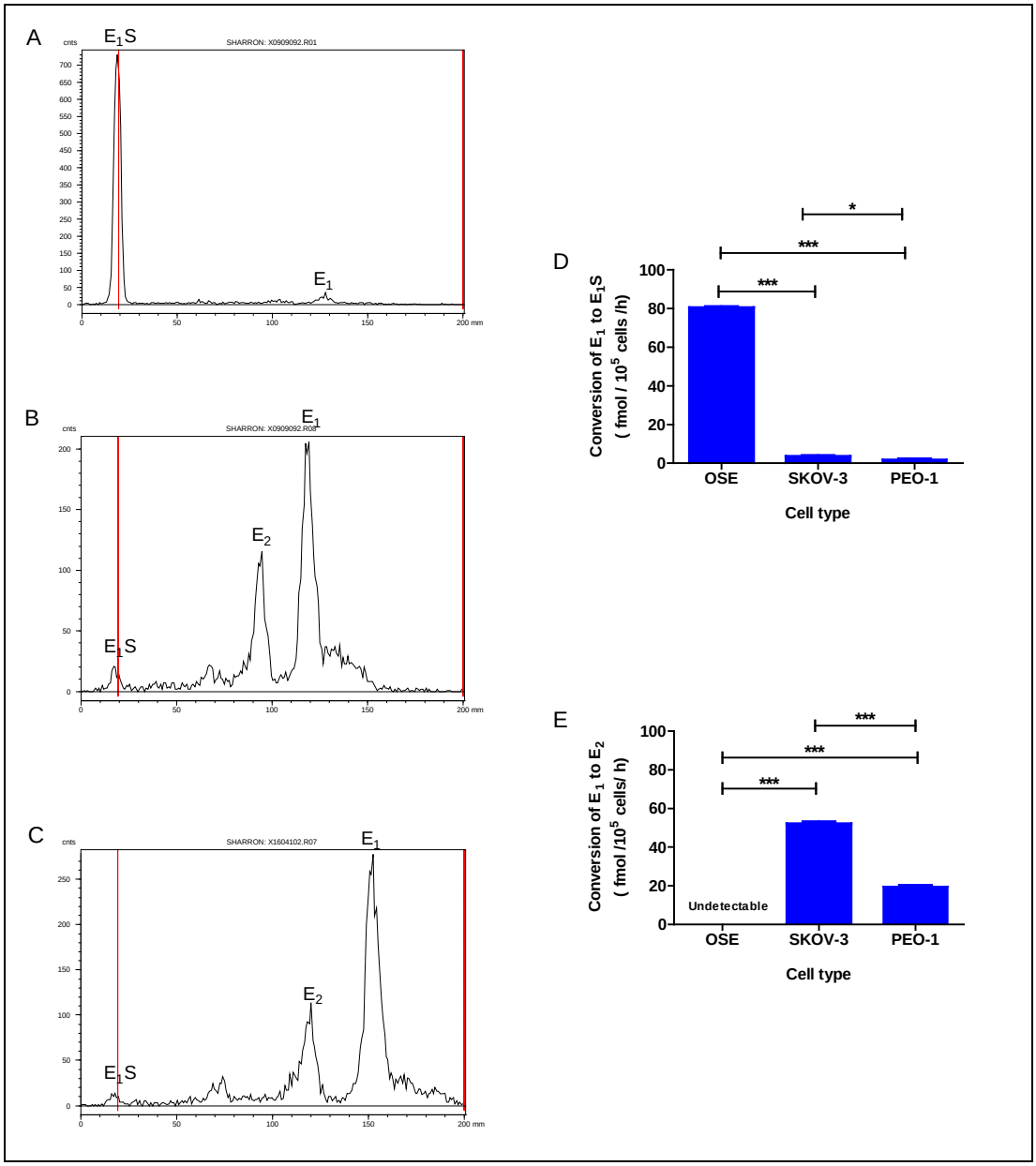

Figure 4.9: Radiochromatographic analysis of oestrogen pre-receptor metabolites in OSE (A, n=10), SKOV-3 (B, n=3) and PEO-1 (C, n=3) cells. Cells were cultured for 72h with [^{3}H]-E_1 and tritium labeled metabolites were detected by TLC and quantified by radioactivity scanner. Representative graphs indicate readout from TLC plates where y-axis shows counts of radioactivity and x-axis demonstrates the distance along the plate. Histograms show conversion rate of E_1 to E_1S (D) and E_1 to E_2 (E) calculated as described in Section 2.5. Bars indicate Mean±SEM. (*=P<0.05, ***=P<0.001)

4.3.3.2.2 Effect of Glusulase on [^{3}H]-E_1 metabolism in OSE cells

To confirm that the majority of E_1 radiometabolite was at the position of E_1S, OSE cell extracts were incubated with an exogenous source of STS activity-Glusulase (Glu) and radio chromatography was then carried out. 500,000 cells were plated and serum-depleted for 24h before treatment. After treatment with [^{3}H]-E_1 for 72h, medium was collected and tritium-labelled steroids were extracted as described in Section 2.5. The dry steroid extract was then reconstituted in buffer with or without Glu and kept in 37°C waterbath for 2h. The tubes were shaken every 15min to ensure a full reaction. The reaction was then stopped by adding 10mL dichloromethane for the extraction of the steroids. The subsequent steps were as described for the normal enzyme activity assay (Section 2.5). The OSE cells without Glu converted most of [^{3}H]-E_1 back to the original position of the [^{3}H]-E_1S (Fig. 4.10, A). The OSE cells from the same patient with Glu converted the above product back to the [^{3}H]-E_1 position (Fig. 4.10, B). When the radiographs were quantified as described in Section 2.5, the conversion rate of E_1 to E_1S was significantly decreased by Glu (Fig. 4.10, **=$P<0.005$).

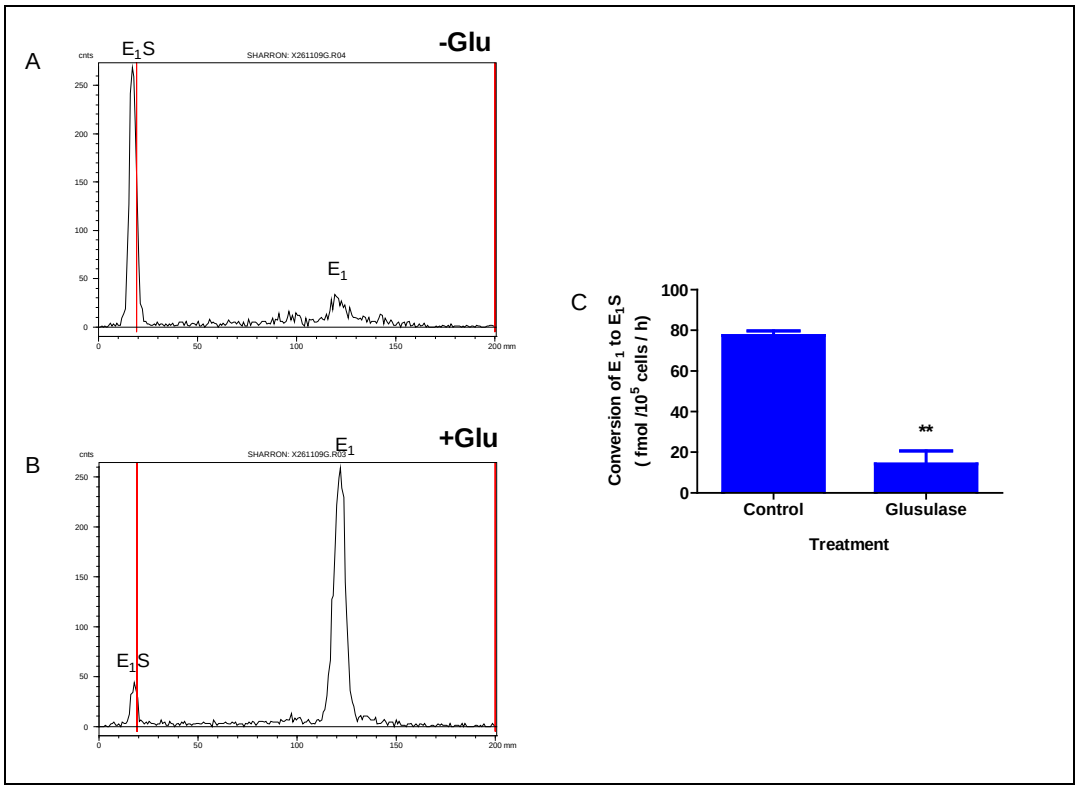

Figure 4.10: Effect of Glusulase on oestrogen pre-receptor metabolites in OSE cells. Cells were cultured for 72h with [^{3}H]-E_1 and then treated further without (A) or with (B) Glu. Tritium labeled metabolites were detected by TLC and quantified by radioactivity scanner. Representative graphs indicate readout from TLC plates where y-axis shows counts of radioactivity and x-axis demonstrates the distance along the plate. Histograms show conversion rate of E_1 to E_1S (C) calculated as described in Section 2.5. Bars indicate Mean±SEM. (n=3, P<0.005)

4.4 Discussion

In Chapter 3, we showed by IHC that STS, EST, 17βHSD2 and 17βHSD5, but not aromatase proteins are present in OSE of pre-menopausal ovary, post-menopausal ovary and inclusion cysts as well as EOC. In the present chapter, we investigated the genes encoding the metabolism and action of oestrogens further by comparing the mRNA expression of target genes in pre-menopausal OSE, EOC cells and ovarian cancer cell lines and studying activities of the encoded enzymes in pre-menopausal OSE cells and ovarian cancer cell lines.

Similar STS mRNA expression in both OSE and EOC cells is consistent with the IHC result. Moreover STS mRNA was detected in PEO-14, SKOV-3 and PEO-1 cell lines and the mRNA levels are similar to OSE and EOC cells. Therefore, data from both primary cells and cancer cell lines are consistent with the possibility of E_1 synthesis from E_1S in the normal and cancer cells by STS pathway, but suggest that any differences between normal OSE and cancer tissue is not determined at mRNA expression level. Since STS can also convert DHEAS to DHEA (a precursor of androgen) and 3βHSD (the enzyme responsible for the conversion of DHEA to A) is present in OSE and EOC (Papacleovoulou, Edmondson et al. 2009), it is still possible that some E_1 and E_2 are produced by the aromatase pathway in the samples which have aromatase expression and function, although given the low aromatase expression observed (see below) this seems unlikely to be a major pathway.

In keeping with the tissue protein expression (Chapter 3), aromatase mRNA expression in OSE and EOC cells was roughly one thousand times lower than in placenta. This could explain why aromatase protein was detected in placenta tissue but not in normal ovary and ovarian cancer tissues run in the same round using the same concentration of primary antibody. Moreover its expression was undetectable in 6 OSE samples and there was no difference in expression between OSE and EOC cells. Among 3 cell lines tested, aromatase mRNA was only present in PEO-14 and not in SKOV-3 and PEO-1 cells, which was in agreement with the study performed

by Cunat (Cunat, Rabenoelina et al. 2005). Compared with STS mRNA and protein expression which were detected in all normal and cancer tissues, cells, and cell lines, aromatase mRNA and protein expression was not widely expressed and was undetectable in some samples.

In contrast to STS mRNA expression, EST mRNA was much lower in EOC than in OSE cells with undetectable expression in half of the EOC samples. Additionally, EST mRNA expression was much lower in PEO-14 cells compared with OSE cells and was undetectable in PEO-1 and SKOV-3 cells, further confirming that EST mRNA expression was lower in ovarian cancer cells compared to normal cells. However, there was no difference in EST protein expression detected by IHC (Chapter 3), which conflicts with the mRNA data. One explanation might be that mRNA is only measuring the gene expression and does not always reflect the expression of the protein, which can be affected by mRNA stability and post-translational modifications. This lower mRNA expression of EST in cancer cells indicated there is less protection against oestrogenic effects in EOC and therefore there would likely be more active oestrogen produced in cancer cells locally, providing an environment that is pro-tumourigenic.

The pattern of expression of the 17βHSD genes observed in the cancer cell lines, OSE and EOC were consistent with the IHC results in which 17βHSD2 and 17βHSD5 were expressed in not only OSE but also EOC cells. QRT-PCR confirmed 17βHSD1 mRNA was expressed in most OSE and EOC samples, although we did not look for 17βHSD1 protein expression by IHC. The interconversion of E_1 and E_2 is a bi-directional equilibrium reaction depending on the balance of 17βHSD1/5 and 17βHSD2, and the amount of the E_2 synthesized is determined by the level and activity of these enzymes. mRNA expression of 17βHSDs suggested again the possibility of the local production of E_2 from E_1 in OSE and EOC cells and the lack of difference in the 17βHSDs gene expression between OSE and EOC indicated the similar production of E_2 production between the normal and cancer cells, although we have not tested their activities to finally determine the production of E_2 by these

cells. Our results complement previous studies which have demonstrated 17βHSDs expression in normal and malignant ovaries. 17βHSD1 immunoreactivity was absent in the surface epithelium of the normal cycling human ovary and in tumour cells of 12 benign cystadenomas while it was present in the cytoplasm of four out of eight specimens of low malignant potential and in 20 out of 30 specimens of invasive carcinoma (Sasano, Frost et al. 1996). Significant levels of 17βHSD1 and 17βHSD2 mRNAs were observed in an ovarian cancer OV-90 cell line (Provost, Lima et al. 2010).

Studies of breast cancer demonstrated that 17βHSDs could be prognostic factors in breast cancer. Oduwole showed breast cancer patients with tumours expressing 17βHSD1 mRNA or protein had significantly shorter overall and disease-free survival than the other patients. Moreover, the expression of 17βHSD5 was significantly higher in breast tumour specimens than in normal tissue. The group with 17βHSD5 over expression had a worse prognosis than the other patients (Oduwole, Li et al. 2004). Similarly, Gunnarsson reported ER positive patients with high expression levels of 17βHSD1 had a significantly higher risk for late relapse and ER-positive patients with low 17βHSD2:17βHSD1 ratio showed a significantly higher rate of recurrence than those with higher ratios (Gunnarsson, Hellqvist et al. 2005). The relationship between the expression and prognosis of 17βHSDs in ovarian cancer has not been studied. We would predict that this relationship in ovarian cancer is similar to breast cancer, and ovarian cancer patients with higher 17βHSD1 and 17βHSD5 or lower 17βHSD2 expression would have a poor prognosis, but this remains to be determined.

To complement the above mRNA studies, protein expression was further assessed by functional studies of enzymic activity. STS and 17βHSD1/5 convert E_1S to E_2 *in vivo* while EST and 17βHSD2 have the opposite effect. In addition, there is the possibility that EST converts E_2 to E_2S. All the above actions result in equilibrium between these enzymes. Studies using tritiated E_1S or E_1 as substrate give a direct indication of the direction and extent of metabolism of E_1S or E_1 in normal and cancer cells. As expected, there was a clear cut difference in the direction of E_1S and E_1 metabolism

between OSE and ovarian cancer cell lines. When E_1S was added as the substrate, SKOV-3 and PEO-1 cells converted E_1S to E_1 and then to E_2 while there was no E_1 or E_2 produced by OSE cells. Conversely, when E_1 was added as substrate, SKOV-3 and PEO-1 cells converted E_1 to E_2, while there was no E_2 produced in OSE cells and most of E_1 was converted to E_1S or E_2S at 72h. The difference can be explained by the mRNA expression of these genes. There was STS, 17βHSD1 and 17βHSD5 but no EST and 17βHSD2 mRNA expression in SKOV-3 cells. Therefore the metabolism of E_1S to E_1 in SKOV-3 cells could indicate active STS and the metabolism of E_1 to E_2 could suggest active 17βHSD1/5. There was STS, 17βHSD5 and 17βHSD2 but no EST mRNA expression in PEO-1 cells. Therefore the metabolism of E_1S to E_1 in PEO-1 cells could indicate active STS and the metabolism of E_1 to E_2 could suggest 17βHSD5 was more active than 17βHSD2. mRNA of STS, 17βHSD1/5 and EST, 17βHSD2 are all expressed in OSE cells. Therefore the metabolism of E_1S and E_1 would be in equilibrium between these five enzymes if the mRNA expression was reflected in enzyme activity. When E_1S was added as substrate, there were no peaks of E_1 and E_2 radioactivity but there was radioactivity peak at the place where the E_1S peak should be. However the experiment could not specify which activity peak it was. There were two possibilities. It could be E_1S peak if EST and 17βHSD2 were more active than STS and 17βHSD1/5. It could be E_2S peak if STS and 17βHSD1/5 were active and E_2 has higher binding affinity with EST than E_1. It would be interesting to clarify what the final products metabolized from E_1S and E_1 were, by investigating the binding affinity of E_1 and E_2 to EST and kinetics of these enzymes. Nevertheless, both E_1S and E_2S are oestrogen sulphates, conjugated estrogens that do not bind to ER and can not activate the ER pathway. Overall, these enzymatic studies confirm that STS, 17βHSD1 and 17βHSD5 are active in some cancer cells to produce E_2. In addition, 17βHSD5 is more active than 17βHSD2 in some cancer cells, favouring oestrogen activation in the cancer cells. EST is more active than STS in OSE cells, favouring oestrogen inactivation in normal cells.

In the enzymatic assay, it would be arguable 72h was not the optimum experimental time point to test the enzyme activity, since the cells might not grow well after being cultured without serum for four days and enzyme degradation might occur. In addition, because the metabolism was potentially bidirectional, 72h was long enough to allow the reverse enzymes to act if the activites of only STS and 17βHSD1/5 were investigated. Therefore it would be not clear if the target enzymes were active or not. Before 72h was chosen as the experimental time, one time course enzymatic assay experiment, including 12h, 24h, 48h and 72h (data not shown) was performed on SKOV-3 cells. The production of E_1 was seen at 48h but the formation of E_2 was detected only at 72h. Additonally, the cells were checked before the medium was taken out and they appeared morphologically normal. However it would be of interest to perform a short-term time course including 1h, 2h and 4h time points in SKOV-3 and PEO-1 cells and also on OSE if more cells were available.

ERα mRNA was expressed in all OSE and EOC and ERβ was detected in about half of OSE and all EOC samples. This is similar to the expression detected in human OSE expressed ERα and ERβ mRNA by Southern analysis (Hillier, Anderson et al. 1998). Lau observed co-expression of ERα and ERβ mRNA along with AR and PR transcripts in normal human OSE cells (Lau, Mok et al. 1999). However, in contrast, Brandenberger found only ERα mRNA expression in IOSE-Van, an immortalized human OSE cell line (Brandenberger, Tee et al. 1998). Data from this report cannot be directly compared with the current findings because IOSE-Van is a human OSE cell line immortalized by SV40 and therefore the ER characteristics may be different from primary OSE cells. The current study did not show any difference in ERα and ERβ mRNA expression between OSE and EOC cells. Similarly, Lau found variable ERα mRNA expression in ovarian cancer cells and levels of ERβ mRNA were unaffected by malignant status (Lau, Mok et al. 1999). However, using Northern blotting techniques, Brandenberger found ERβ mRNA levels were markedly decreased in ovarian cancer tissue (Brandenberger, Tee et al. 1998) and Bardin found a marked decrease in ERβ mRNA levels in ovarian cancers compared with normal ovarian tissues by qRT-PCR (Bardin, Hoffmann et al. 2004). The explanation for this

discrepancy may be that Brandenberger and Bardin tested ER expression in normal ovary and ovarian cancer tissues which included not only OSE and EOC cells but also stromal or other cell types such as granulosa cells which express high levels of ERβ (Enmark, Pelto-Huikko et al. 1997) while Lau and the current study compared only OSE and EOC cells. Moreover, IHC experiments have demonstrated that ERβ protein levels are lower in ovarian tumours compared to normal ovary (Fujimura, Hidaka et al. 2001; Li, Baldwin et al. 2003; Lindgren, Cajander et al. 2004). Furthermore, there is decreased expression of ERβ in different cancers, such as breast cancer (Roger, Sahla et al. 2001), prostatic cancer (Horvath, Henshall et al. 2001), lung cancer (Stabile, Davis et al. 2002) and colorectal cancer (Foley, Jazaeri et al. 2000). The mechanisms accounting for the decreased expression of ERβ in tumours remain elusive. Although it is still debatable which ER play a more important role in ovarian cancer, the finding that ERα and ERβ transcripts are expressed in OSE and EOC cells suggests that oestrogen, acting through one of its nuclear receptors, may play an important role in regulating oestrogen action in both normal and cancer cells.

ERα mRNA expression is much higher in PEO-1 and SKOV-3 cell lines compared to OSE, EOC and PEO-14 cells, although there is no apparent difference of ERβ mRNA levels between these cells. The high ERα and detectable ERβ mRNA expression in SKOV-3 cell line would indicate there should be an oestrogenic effect on the growth of the cells. It is therefore intriguing that Hua found the growth of SKOV-3 cells was not influenced by E_2 or the anti-oestrogens hydroxy-tamoxifen or ICI 164,384, despite the presence of functional ERs. The explanation for the oestrogen resistance may be a result of constitutive expression and loss of E_2 regulation of selected growth regulatory genes such as HER-2/*neu* and cathepsin D (Hua, Christianson et al. 1995). Since SKOV-3 cells do not respond to E_2, the current study used PEO-1 rather than SKOV-3 cell line as the oestrogen-responsive ovarian cancer model to study the effect of E_2 in ovarian cancers (Chapter 5).

Since E_1S does not easily cross the plasma membrane by diffusion, transporters are important for the passage of E_1S into the cells. The expression of different transporters may affect the net production of E_2, and therefore it is important to investigate their expression in normal ovary and ovarian cancer. Transporters have been studied in most detail in breast cancers and cell lines derived from them. Nozawa demonstrated OATP-D and OATP-E mRNA expression in one breast cancer cell line T-47D by RT-PCR (Nozawa, Suzuki et al. 2004). Nozawa also revealed OATP-D and OATP-E mRNA expression in another breast cancer cell line MCF-7 and found inhibition of E_1S transporter suppressed the transcription and cell proliferation induced by E_1S in MCF-7 cells (Nozawa, Suzuki et al. 2005). Al Sarakbi found mRNA of OATP-B was expressed in both malignant breast cancer and normal breast tissues, and OATP-B mRNA level was correlated with the grade and stage of the disease, but not with the clinical outcome (Al Sarakbi, Mokbel et al. 2006). However, the expression of E_1S transporters in normal and malignant ovaries is still poorly characterized. To the best of my knowledge this is the first study of the E_1S transporters in OSE and EOC. Similar to breast cancer, OATP-B, OATP-D and OATP-E mRNAs were expressed in both OSE and EOC cells. This suggests the possibility of E_1S uptake by OSE and EOC cells via these transporters. Interestingly, OATP-B mRNA was much higher in EOC than in OSE cells, indicating more E_1S could be transported into cancer cells than into normal cells, potentially resulting in more production of E_2. We also tested mRNA levels of OAT4 which was proved to be an important E_1S transporter in placenta (Ugele, Bahn et al. 2008) but the expression was much lower than the placenta standard and undetectable in most of OSE and EOC cells, suggesting a less important role of OAT4 than the other three transporters. The study provides the initial evidence that OATP-B, OATP-D and OATP-E are important E_1S transporters in normal and malignant ovarian cells, indicating a potential novel strategy for ovarian cancer treatment by blocking the transporters. In breast cancer bromosulfophthalein was demonstrated to be an effective E_1S transporter inhibitor and significantly inhibited transcription of reporter gene via oestrogen response element and cell proliferation induced by E_1S (Nozawa, Suzuki et al. 2005). Thus it would be interesting to investigate the effect of transporter inhibitors on the development of EOC. For example, the expression of

E_1S transporters in SKOV-3 and PEO-1 cells can be tested, relevant inhibitors of E_1S transporters can be then tried to invstigate its effect on the local formation of E_2 and proliferation or migration of ovarian cancer cells.

It could be argued that pre-menopausal OSE is an inappropriate cell type to use for comparison with EOC because the OSE was collected from pre-menopausal women whereas most of the ovarian cancers were collected from post-menopausal women. Thus the steroid backgrounds are likely to have been quite different for both tissues. Nevertheless, in terms of the primary nature of EOC cultures, primary OSE is more relevant as a comparator than OSE cell lines. Since there are few post-menopausal women undergoing operations for benign diseases, it is more difficult to get enough post-menopausal OSE, and therefore it is only feasible to use pre-menopausal OSE.

One weakness of the study is the limited numbers of primary OSE and EOC samples for comparison, which decreases the strength of statistical analysis. It could have been improved by increasing the numbers of primary samples used. However primary tissue is a rare and valuable resource and the OSE samples are limited.

In conclusion, in Chapter 4, we have demonstrated STS, EST, aromatase, 17βHSD1, 17βHSD2, 17βHSD5, ERα, ERβ, OATP-B, OATP-D, OATP-E and OAT4 mRNA are expressed in pre-menopausal OSE, EOC and ovarian cancer cell lines at different levels. There is no significant difference in mRNA expression of these target genes between pre-menopausal OSE and EOC, except EST mRNA, which is significantly lower in EOC compared to pre-menopausal OSE cells, and OATP-B which is much higher in EOC compared to pre-menopausal OSE cells. Moreover, STS and 17βHSD1 or 17βHSD5 proteins are active in SKOV-3 and PEO-1 cells, resulting in the production of E_2 in cancer cells when E_1S or E_1 is added as substrate. EST protein is more active than STS protein, resulting in the production of estogen sulphates and preventing formation of E_2 in pre-menopausal OSE cells. The evidence presented indicates that E_2 could be produced locally from circulating conjugated oestrogens by cancer cells, through activity of these oestrogen metabolizing enzymes

and therefore may be an important factor in the development of ovarian cancer even after the cessation of ovarian oestrogen production from developing follicles.

Chapter 5

Inflammatory regulation of oestrogen pathway genes in pre-menopausal OSE, EOC and ovarian cancer cell lines

5.1 Introduction

As stated in Chapter1, factors that decrease a woman's lifetime ovulation rate like pregnancy, multiparity, breastfeeding and oral contraception are associated with a reduced risk of ovarian cancer, suggesting that ovulation is linked to ovarian cancer. In addition, ovulation is proposed to be an acute inflammatory reaction (Espey 1980). Therefore ovulation-associated inflammation may link with ovarian cancer. Moreover, various initiators of local epithelial inflammation, such as PID, talc and asbestos exposures and endometriosis are proposed to contribute to ovarian carcinogenesis (Ness and Cottreau 1999). Taken together, this evidence suggests inflammation plays an important role in the pathogenesis and development of ovarian cancer. In this regard, studies focusing on how inflammation regulates oestrogen metabolizing genes could identify potential diagnostic or therapeutic targets for ovarian cancer.

Around the time of ovulation, a number of cytokines are secreted not only from OSE itself but also from follicular and stromal cells. It is therefore quite reasonable to speculate that several cytokines could have paracrine or intracrine roles in inflammation-associated regulation of oestrogen pathway genes. For example, IL-1α, IL-6 and IL-8 that are released before ovulation, are pro-inflammatory cytokines and might well affect local steroid biosynthesis during ovulation. Moreover, IL-4 and IL-10 are secreted peri- and post-ovulatory and might well mediate intracrine formation of steroids that mediate post-ovulatory responses. It is also of interest that all these cytokines or at least their receptors have been identified in the ovarian tumour microenviroment, suggesting that they may exert a tumourigenic or anti-tumourigenic role in EOC, with the potential for implications in the treatment of the disease.

In Chapters 3 and 4, we established that STS, EST, 17βHSD2 and 17βHSD5 mRNA and protein were present in OSE and EOC, and that E_2 could be produced locally in cancer cells through the activity of these enzymes. In addition, ERα protein was

shown to be expressed in OSE and EOC previously and thus locally produced E_2 could activate the post-receptor pathway and act as a cancer-stimulating agent via its receptor. Consistent with its cancer-promoting action, it is predicted that during periods of inflammation, oestrogen pathway genes would be regulated by cytokines. The regulation of, production and action of E_2 and ER by cytokines are consequently of interest.

The hypothesis is that inflammation can regulate oestrogen metabolizing enzymes and ER expression in OSE or EOC and thus have a role in the development of ovarian cancer. Hence the aim of this chapter is to investigate regulation of STS, EST, 17βHSD2, 17βHSD5 and ERα by inflammatory and anti-inflammatory cytokines IL-1α, IL-4, IL-6, IL-8 and IL-10 in OSE, EOC cells and ovarian cancer cell lines.

5.2 Materials and methods

5.2.1 OSE, EOC cells and SKOV-3 cell line

Pre-menopausal OSE and EOC cells were collected and cultured as described in Sections 2.1.1, 2.1.2 and 2.2.1. The cells were plated out as described in Sections 2.2.2, treated with different cytokines and the RNA was collected as stated in Section 2.4.1.1. The basic clinical profiles of patients involved in the respective assays are presented in Table 5.1 below. Information on SKOV-3 cells was listed in Section 2.2.1.

Patient No	**Code**	**LREC No**	**Age**	**Reason for surgery**	**Surgery**	**Study**
1	6321	04/S1103/36	26	Pelvic pain	DiagLapar	qRT-PCR
2	6800	04/S1103/36	50	Fibroids	TAHBSO	qRT-PCR
3	7527	04/S1103/36	38	Prophylactic	LapSter	qRT-PCR
4	7538	04/S1103/36	21	Pelvic pain	DiagLapar	qRT-PCR
5	7543	04/S1103/36	26	Prophylactic	LapSter	qRT-PCR
6	7548	04/S1103/36	31	Prophylactic	LapSter	qRT-PCR
7	7549	04/S1103/36	29	Prophylactic	LapSter	qRT-PCR
8	7565	04/S1103/36	32	Pelvic pain	DiagLapar	qRT-PCR
9	7566	04/S1103/36	41	Pelvic pain	DiagLapar	qRT-PCR
10	7606	04/S1103/36	36	Prophylactic	LapSter	qRT-PCR
11	7613	04/S1103/36	48	Fibroids	TAH	qRT-PCR
16	CA11	04/S1103/36	N/A	N/A	N/A	qRT-PCR
17	CA9B	04/S1103/36	N/A	N/A	N/A	qRT-PCR
18	44021	04/S1103/44	55	EOC	N/A	qRT-PCR
19	44028	04/S1103/44	59	EOC	N/A	qRT-PCR
20	44029	04/S1103/44	67	EOC	N/A	qRT-PCR
22	3028	04/S1103/36	29	Pelvic pain	DiagLapar	TLC

Patient No	Code	LREC No	Age	Reason for surgery	Surgery	Study
24	6316	04/S1103/36	43	HMB and pelvic pain	TAHBSO	TLC
25	6318	04/S1103/36	41	Hydrosalpinx	Romoval of Hydrosalpinx	TLC
3	7527	04/S1103/36	38	Prophylactic	LapSter	TLC
4	7538	04/S1103/36	21	Pelvic pain	DiagLapar	TLC
26	7541	04/S1103/36	33	Prophylactic	LapSter	TLC
27	7544	04/S1103/36	34	Prophylactic	LapSter	TLC
28	7546	04/S1103/36	19	Left ovarian cyst	DiagLapar	TLC
29	7550	04/S1103/36	31	Prophylactic	LapSter	TLC

Table 5.1: Clinical profile of patients used for qRT-PCR and TLC.

DiagLapar: diagnostic laparoscopy; EOC: epithelial ovarian cancer; LapSter: laparoscopic sterilization; HMB: heavy menstruation bleeding; LREC: Lothian Research Ethical Committee; N/A: not available; TAH: total abdominal hysterectomy; TAHBSO: total abdominal hysterectomy and bilateral salpingo-oophorectomy; TLC: thin layer chromatography.

5.2.2 RNA extraction and reverse transcription

RNA was extracted using methods described in Section 2.4.1 and RT-PCR was performed using standard conditions as described in Section 2.4.2.

5.2.3 Taqman qRT-PCR

QRT-PCR was performed using an ABI 7900 sequence detection system and analysed as described in section 2.4.3. The probes and primers used were commercially available pre-validated from Applied Biosystems Assay on Demand or designed in house. The information of the primers and probes is shown in Table 2.3.

5.2.4 Enzyme activity assay

The activities of the enzymes encoded by the target genes were tested using the method described in Section 2.5.

5.2.5 Statistical analysis

Statistical analysis was performed using GraphPad Prism 5. QRT-PCR data in Section 5.3.1 were analyzed by Wilcoxon matched pairs test. QRT-PCR data in Section 5.3.2.1 and 5.3.2.3 were analyzed by one-way ANOVA with Tukey post-hoc test. QRT-PCR data in Section 5.3.2.2 were analyzed by two-way ANOVA. Data from enzyme activity assays in Section 5.3.3 were analyzed by T test. QRT-PCR data in Section 5.3.4.1 were analyzed by Kruskal-Wallis test with Dunn's post-hoc test. QRT-PCR data in Section 5.3.4.2 were analyzed by one-way ANOVA with Tukey post-hoc test. Statistical difference was assigned at $P<0.05$.

5.3 Results

5.3.1 Effect of IL-1α on STS, EST, 17βHSD2, 17βHSD5 and ERα mRNA expression in pre-menopausal OSE and EOC cells

Treatment of 13 individual OSE and 3 EOC cell monolayers with 0.5ng/ml IL-1α for 48h resulted in different response of target genes. IL-1α had no effect on STS mRNA expression in either OSE or EOC cells (Fig. 5.1 A, B). Suppression of EST mRNA levels by IL-1α was observed in OSE but not in EOC cells (Fig. 5.1 C, D, $P<0.01$). Moreover, IL-1α inhibited 17βHSD2 mRNA expression significantly in OSE but not in EOC cells (Fig. 5.2 A, B, $P<0.001$) while it had no effect on 17βHSD5 mRNA expression in either OSE or EOC cells (Fig. 5.2 C, D). Furthermore, IL-1α stimulated ERα mRNA expression significantly in OSE (Fig. 5.3 A, $P<0.001$) but had no effect in EOC cells (Fig. 5.3 B).

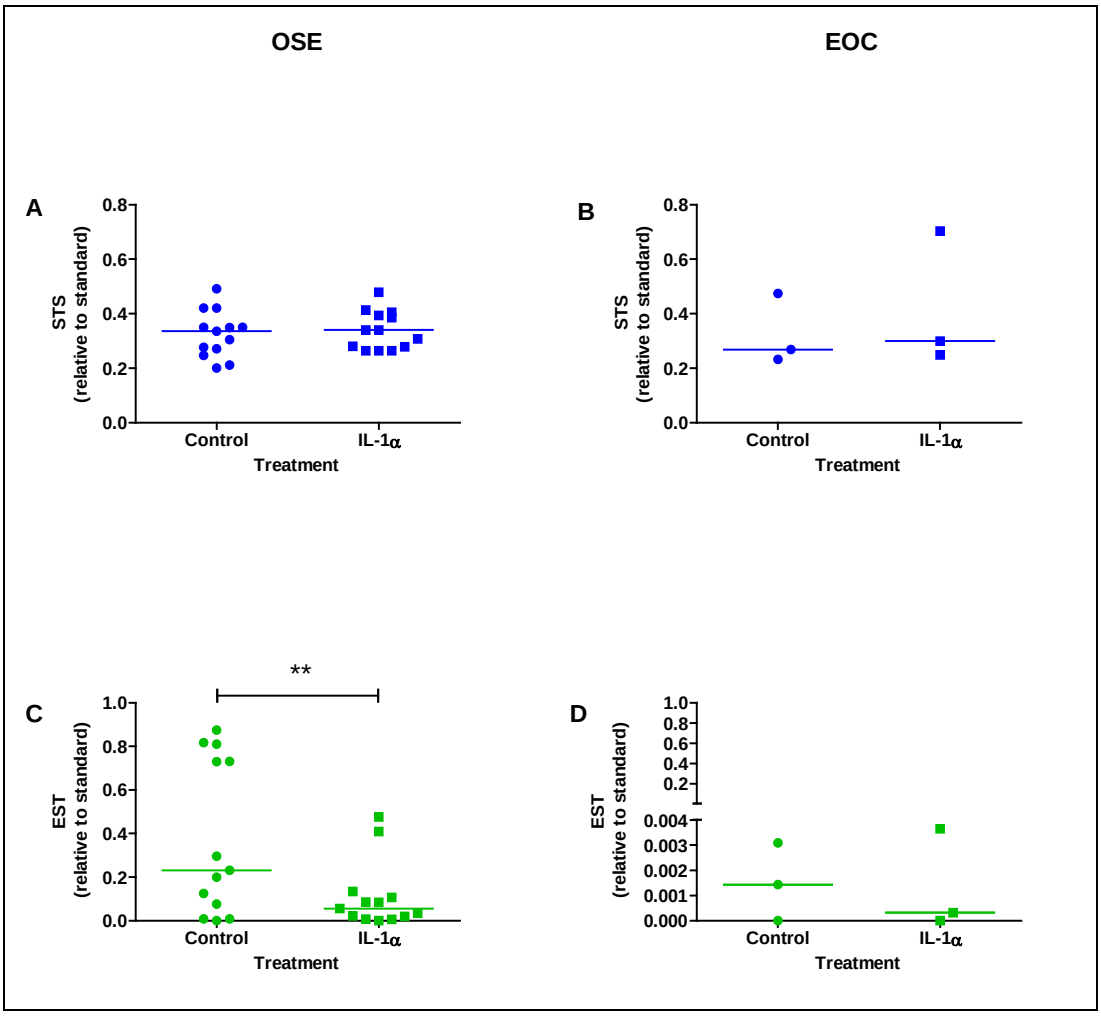

Figure 5.1: Effect of IL-1α on STS and EST mRNA expression in OSE (n=13) and EOC (n=3) cells. OSE and EOC cells were treated with IL-1α (0.5ng/ml) for 48h and expression of STS (A, B) and EST (C, D) mRNA were measured using Taqman qRT-PCR. Horizontal bars indicate median value. (=P<0.01)**

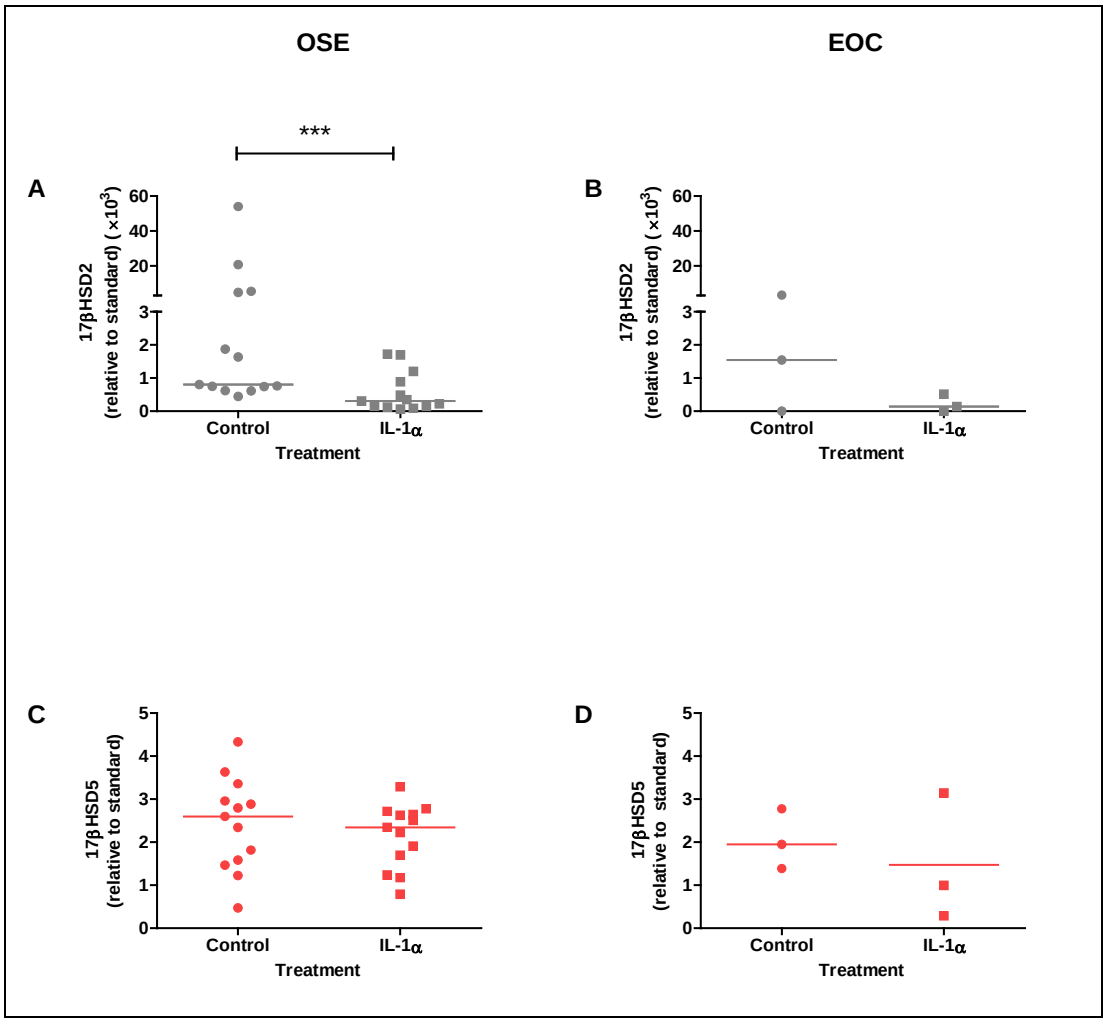

Figure 5.2: Effect of IL-1α on 17βHSD2 and 17βHSD5 mRNA expression in OSE (n=13) and EOC (n=3) cells. OSE and EOC cells were treated with IL-1α (0.5ng/ml) for 48h and expression of 17βHSD2 (A, B) and 17βHSD5 (C, D) mRNA were measured using Taqman qRT-PCR. Horizontal bars indicate median value. (*=P<0.001)**

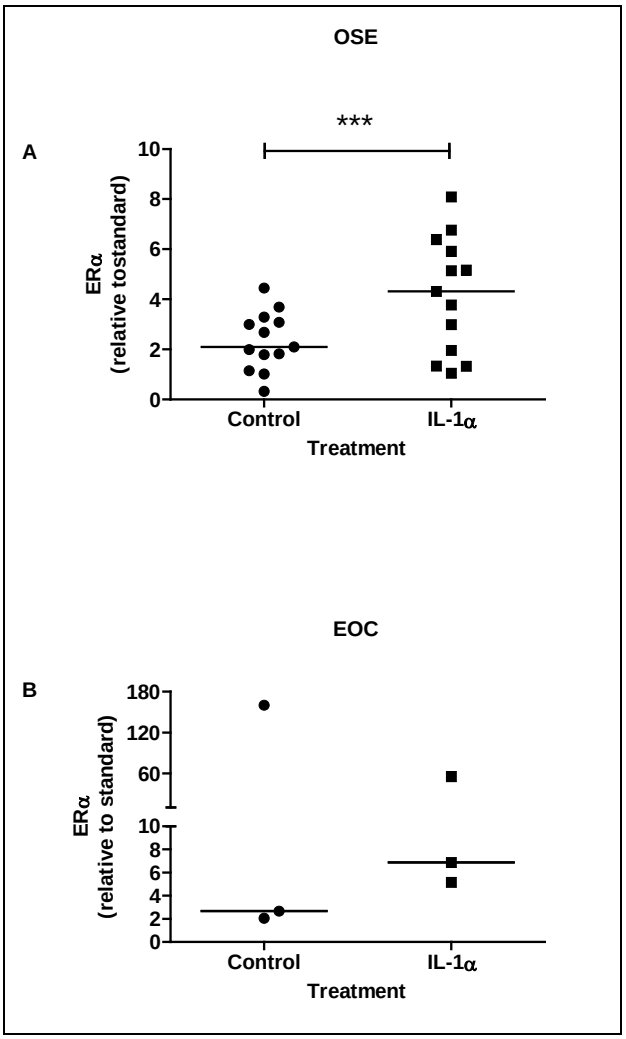

Figure 5.3: Effect of IL-1α on ERα mRNA expression in OSE (n=13) and EOC (n=3) cells. OSE (A) and EOC (B) cells were treated with IL-1α (0.5ng/ml) for 48h and expression of ERα mRNA was measured with Taqman qRT-PCR. Horizontal bars indicate median value. (*=P<0.001)**

5.3.2 Effect of IL-1α on STS and 17βHSD5 mRNA expression in SKOV-3 cell line

5.3.2.1 Dose-dependent effect of IL-1α on STS and 17βHSD5 transcripts

Treatment of SKOV-3 cells with IL-1α *in vitro* for 48h resulted in a dose-dependent stimulation of STS mRNA levels (Fig. 5.4, A). The maximum (2.5-fold) increase occurred when 0.15ng/ml of IL-1α was used (P<0.01). No effect was observed with the lowest concentration tested. On the other hand, IL-1α did not have an effect on 17βHSD5 mRNA at any dose of IL-1α (Fig. 5.4, B).

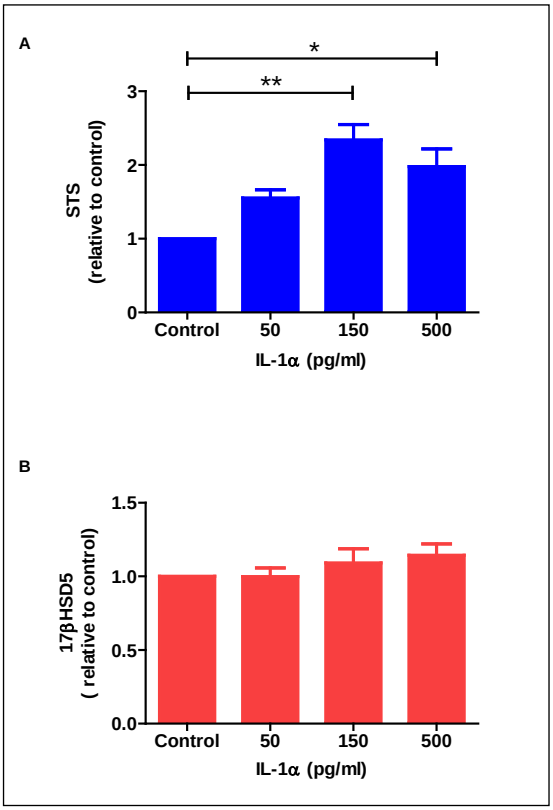

Figure 5.4: Effect of IL-1α on STS and 17βHSD5 mRNA expression in SKOV-3 cells. SKOV-3 cells were treated with increasing doses of IL-1α (0.05-0.5ng/ml) for 48h and expression of STS (A) and 17βHSD5 (B) mRNA were measured with Taqman qRT-PCR. Bars indicate Mean±SEM. Asterisks indicate significant difference from untreated control. (n=3, *=P<0.05, **=P<0.01)

5.3.2.2 Effect of incubation time on IL-1α effect on STS and 17βHSD5 transcripts

Time-course (12, 24, 48h) studies with 0.5ng/ml IL-1α were undertaken to further test its stimulatory effect on STS transcripts (Fig. 5.5A). The stimulatory effect was first observed at the 12h time-point although maximum stimulation was observed at the 48h time-point (Fig. 5.5, P<0.01, P<0.001). IL-1α treatment of OSE cells led to a 2-fold increase of 17βHSD5 mRNA at 12h, which was reduced at 24h and disappeared at 48h (Fig. 5.5, P<0.001).

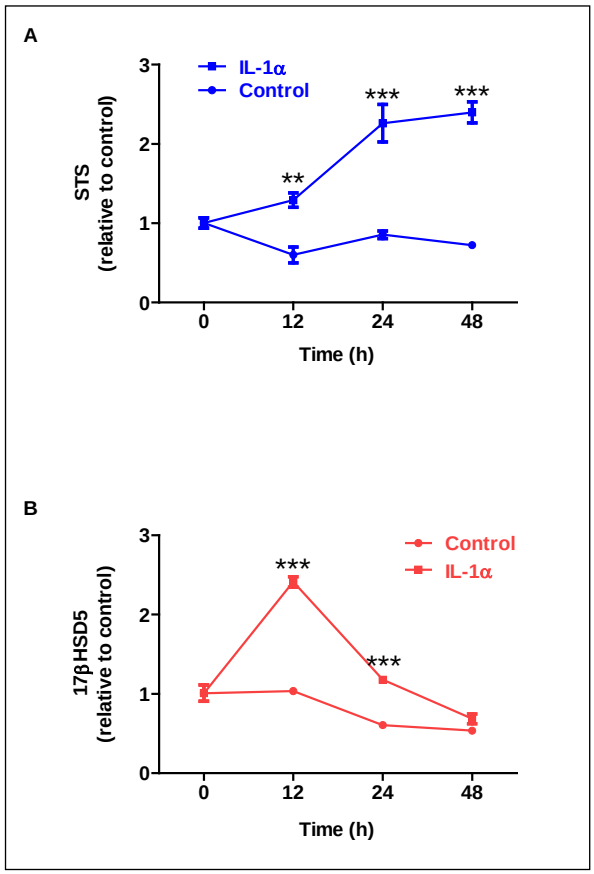

Figure 5.5: Time-dependent effect of IL-1α on STS and 17βHSD5 mRNA expression in SKOV-3 cells. IL-1α treatment (0.5 ng/ml) was applied for increasing panel of time (12, 24, 48h). Expression of STS (A) and 17βHSD5 (B) mRNA were measured by Taqman qRT-PCR. Bars indicate Mean±SEM. Asterisks indicate significant difference from untreated control at the same time point. (=P<0.01, ***=P<0.001)**

5.3.2.3 Involvement of IL-1 receptor in IL-1α-mediated STS mRNA expression

In order to examine if IL-1α exerts its effect on STS mRNA through activation of IL-1 receptor pathway, SKOV-3 cells were treated *in vitro* with IL-1 receptor antagonist (IL-1RA), the inhibitor of IL-1R, in the presence or absence of 0.5ng/ml of IL-1α for 48h. Addition of 25ng/ml IL-1RA reversed IL-1α-elevated STS mRNA level (Fig. 5.6, P<0.001).

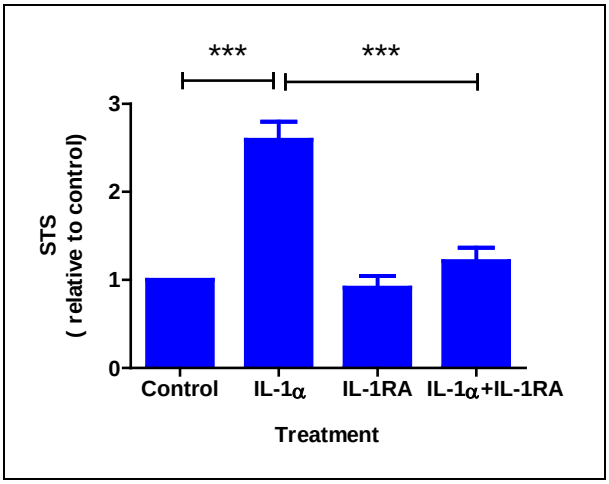

Figure 5.6: Blockade of IL-1α stimulating STS mRNA expression by IL-1RA in SKOV-3 cells. SKOV-3 cells were treated with IL-1α (0.5ng/ml), IL-1RA (25ng/ml) or both for 48h and expression of STS mRNA was measured by Taqman qRT-PCR. Bars indicate Mean±SEM. (n=3, ***=P<0.001)

5.3.3 Effect of IL-1α on metabolism of E_1S in OSE and SKOV-3 cells

After showing that IL-1α inhibited EST and 17βHSD2 mRNA expression in OSE cells and stimulated STS mRNA expression in SKOV-3 cells *in vitro,* the effect of IL-1α on the activity of these enzymes was examined. OSE cells were unable to convert [^{3}H]-E_1S to [^{3}H]-E_1 and [^{3}H]-E_2 even in the presence of IL-1α. In contrast, addition of IL-1α increased conversion of [^{3}H]-E_1S to [^{3}H]-E_1 2-fold in SKOV-3 cells (Fig. 5.7, *=P<0.05), although the effect of IL-1α on the conversion of [^{3}H]-E_1S to [^{3}H]-E_2 was not statistically significant.

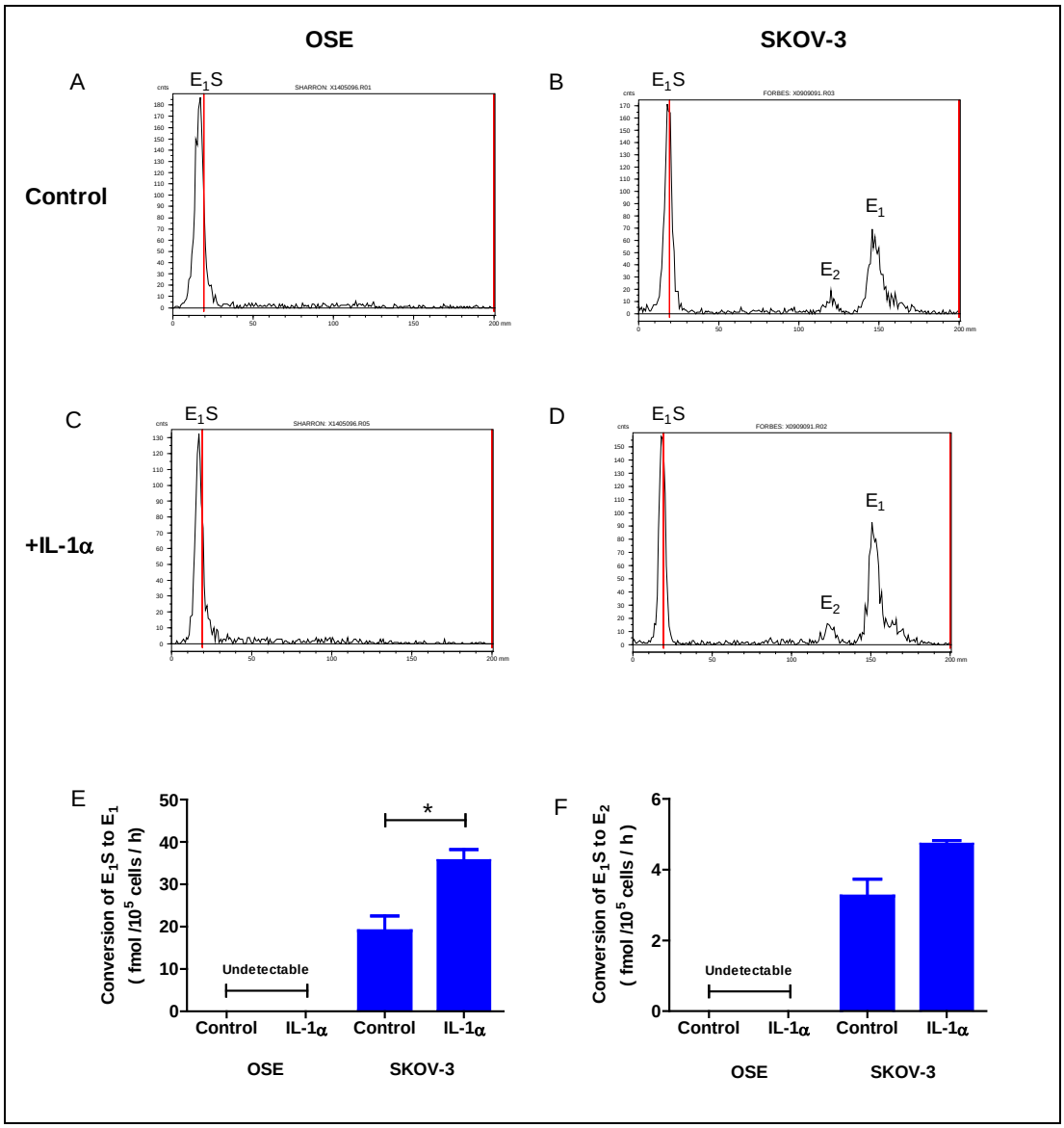

Figure 5.7: Effect of IL-1α on oestrogen pre-receptor metabolizing enzyme activity in OSE (n=9) and SKOV-3 (n=3) cells. 500,000 cells were plated and serum-depleted for 24h before investigating the enzyme activity *in vitro*. [^{3}H]-E_1S was added as a substrate to treat the cells in the presence or absence of 0.5ng/ml of IL-1α for 72h. Radioactive metabolites were separated by TLC and measured by radioactivity scanner. A) Representative OSE sample in the absence of IL-1α; B) Representative SKOV-3 sample in the absence of IL-1α; C) Representative OSE sample after IL-1α treatment (0.5ng/ml); D) Representative SKOV-3 sample after IL-1α treatment (0.5ng/ml). These radiographs indicate readout from TLC plates where y-axis shows counts of radioactivity and x-axis demonstrates the distance along the plate. Different peaks represent E_1S (substrate), E_1 (product of STS) and E_2 (product of STS and 17βHSD5) respectively, as labelled (A-D). Histograms show conversion rate of E_1S to E_1 (E) and E_1S to E_2 (F) calculated as described in Section 2.5. Bars indicate Mean±SEM. (*=P=0.022)

5.3.4 Effect of cytokines on STS, EST, 17βHSD2, 17βHSD5 and ERα mRNA expression in OSE and SKOV-3 cells

5.3.4.1 Effect of cytokines on STS, EST, 17βHSD2, 17βHSD5 and ERα mRNA expression in OSE cells

Having shown the effects of IL-1α on STS, EST, 17βHSD2, 17βHSD5 and ERα mRNA expression, IL-4, IL-6, IL-8 and IL-10 effects were tested in OSE cells from 3 patients. The cells were treated with IL-1α, IL-4, IL-6, IL-8 and IL-10 (all at 0.5ng/ml) for 48h. None of the cytokines had any effect on STS mRNA expression in OSE cells (Fig. 5.8, A). IL-4 reduced EST mRNA level significantly ($P<0.05$), whilst IL-6, IL-8 and IL-10 did not have any effect on EST mRNA (Fig. 5.8, B). Although IL-1α, IL-4 and IL-6 decreased 17βHSD2 mRNA this did not achieve statistical significance. IL-8 and IL-10 did not affect 17βHSD2 mRNA level (Fig. 5.9, A). Although IL-1α inhibited 17βHSD5 mRNA expression and IL-4 enhanced it, again this did not achieve statistical significance. IL-6, IL-8 and IL-10 did not affect 17βHSD5 mRNA level (Fig. 5.9, B). Although IL-1α appeared to stimulate ERα mRNA expression this effect was not statistically significant. IL-4, IL-6, IL-8 and IL-10 did not have any effect on ERα mRNA level (Fig. 5.10).

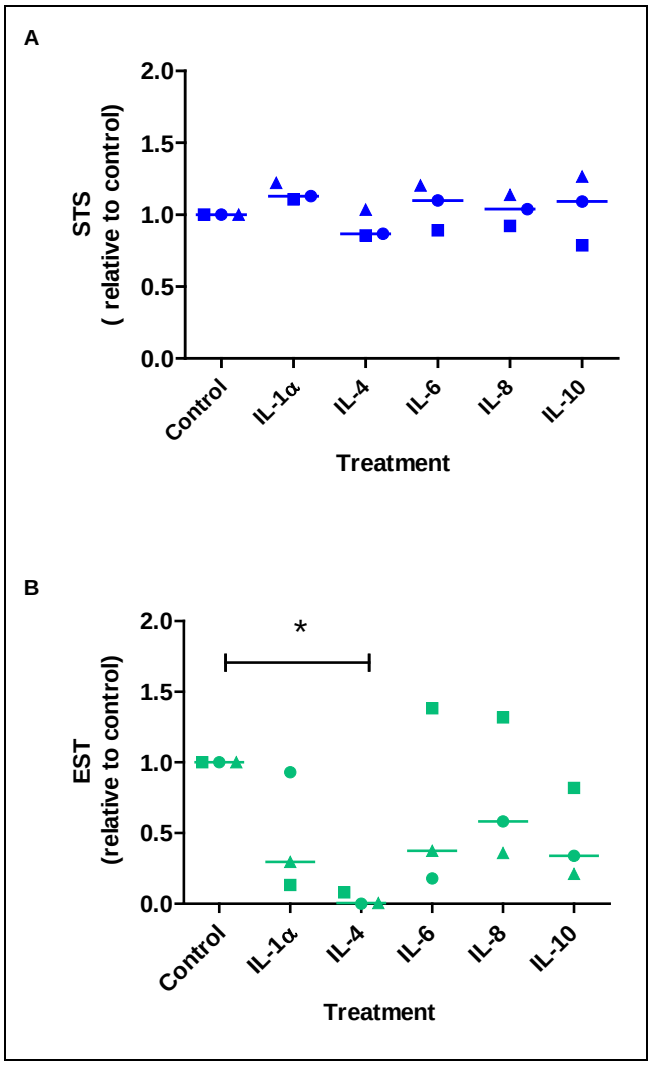

Figure 5.8: Effect of inflammatory cytokines on STS and EST mRNA expression in OSE cells. OSE cells were treated with IL-1α, IL-4, IL-6, IL-8, IL-10 (0.5ng/ml) for 48h and expression of STS (A) and EST (B) mRNAs were measured using Taqman qRT-PCR. Different symbols represent different patients. Horizontal bars indicate median value. Asterisk indicates significant difference from untreated control. (n=3, *=P<0.05)

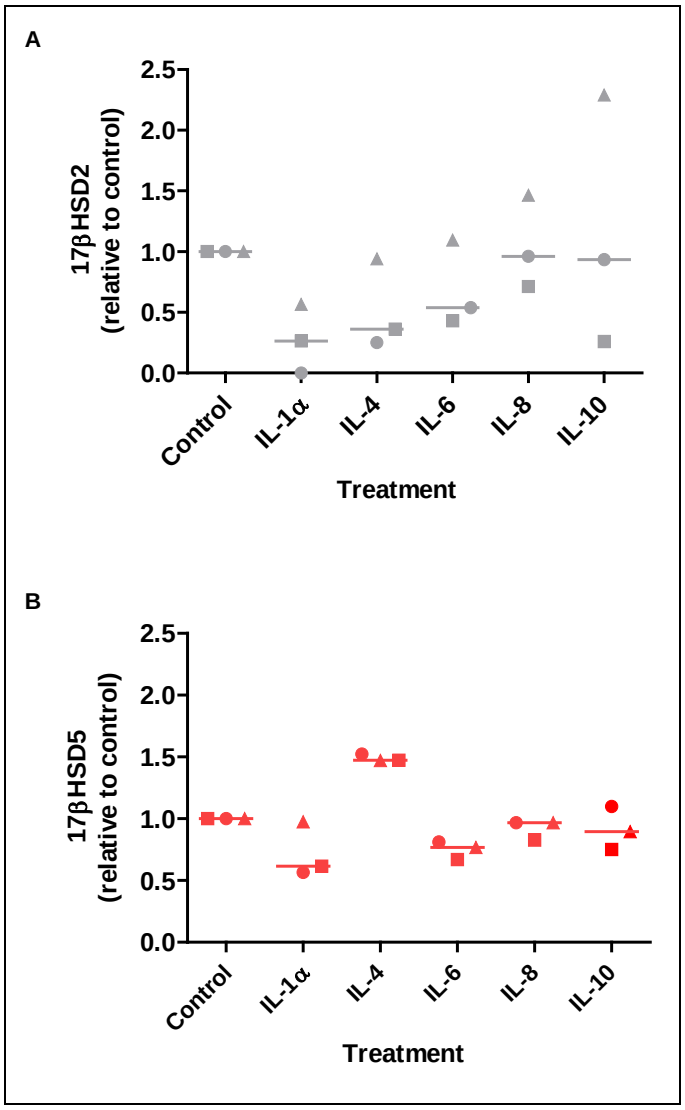

Figure 5.9: Effect of inflammatory cytokines on 17βHSD2 and 17βHSD5 mRNA expression in OSE cells. OSE cells were treated with IL-1*α*, IL-4, IL-6, IL-8, IL-10 (0.5ng/ml) for 48h and expression of 17βHSD2 (A) and 17βHSD5 (B) mRNA were measured using Taqman qRT-PCR. Different symbols represent different patients. Horizontal bars indicate median value. (n=3)

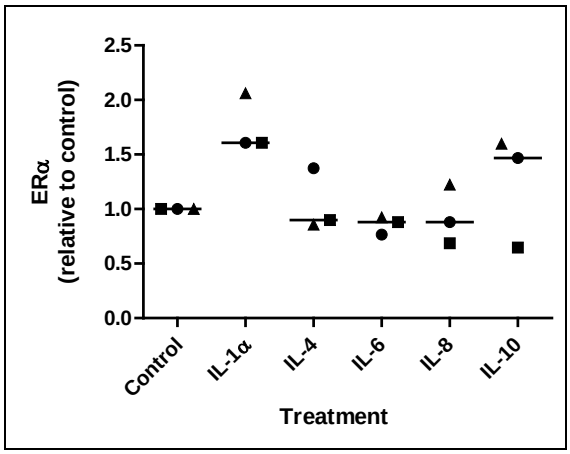

Figure 5.10: Effect of inflammatory cytokines on ERα mRNA expression in OSE cells. OSE cells were treated with IL-1α, IL-4, IL-6, IL-8, IL-10 (0.5ng/ml) for 48h and expression of ERα mRNA was measured using Taqman qRT-PCR. Different symbols represent different patients. Horizontal bars indicate median value. (n=3)

5.3.4.2 Effect of cytokines on STS, 17βHSD5, and ERα mRNA expression in SKOV-3 cell line

Having tested the effect of IL-1α, IL-4, IL-6, IL-8 and IL-10 on STS, EST, 17βHSD2, 17βHSD5 and ERα mRNA expression in OSE cells, the effect of these cytokines on STS, 17βHSD5 and ERα expression in SKOV-3 cells was tested. The cells were treated with IL-1α, IL-4, IL-6, IL-8 and IL-10 (all at 0.5ng/ml) for 48h. Only IL-1α increased STS mRNA expression level significantly which was consistent with the results described in Section 5.3.2 (Fig. 5.11, $P<0.001$). None of the cytokines had any effect on 17βHSD5 mRNA expression (Fig. 5.12). IL-1α and IL-6 did not affect ERα mRNA level while IL-4 inhibited and IL-8 and IL-10 stimulated its expression (Fig. 5.13, $P<0.05$ and $P<0.01$).

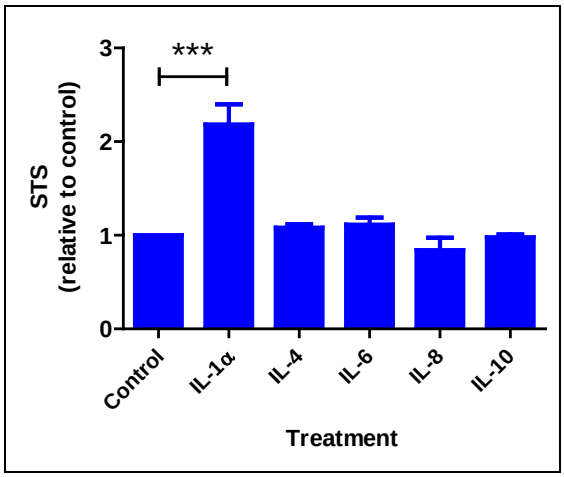

Figure 5.11: Effect of cytokines on STS mRNA expression in SKOV-3 cells. SKOV-3 cells were treated with IL-1α, IL-4, IL-6, IL-8, IL-10 (0.5ng/ml) for 48h and expression of STS mRNA was measured using Taqman qRT-PCR. Horizontal bars indicate Mean±SEM. Asterisk indicates significant difference from untreated control. (n=3, ***=P<0.001)

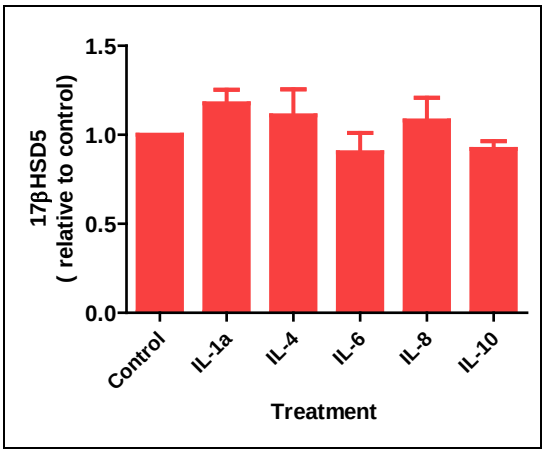

Figure 5.12: Effect of cytokines on 17βHSD5 mRNA expression in SKOV-3 cells. Cells were treated with IL-1α, IL-4, IL-6, IL-8, IL-10 (0.5ng/ml) for 48h and expression of 17βHSD5 mRNA was measured using Taqman qRT-PCR. Horizontal bars indicate Mean±SEM. (n=3)

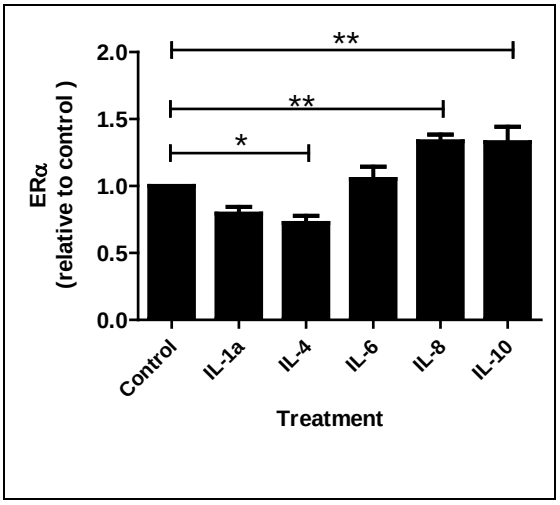

Figure 5.13: Effect of cytokines on ERα mRNA expression in SKOV-3 cells. Cells were treated with IL-1α, IL-4, IL-6, IL-8, IL-10 (0.5ng/ml) for 48h and expression of ERα mRNA was measured using Taqman qRT-PCR. Horizontal bars indicate Mean±SEM. Asterisk indicates significant difference from untreated control. (n=3, *=P<0.05, **=P<0.01)

5.4 Discussion

In Chapters 3 and 4, it was established that STS, EST, 17βHSD2 and 17βHSD5 mRNA and protein are functionally present in OSE and EOC. The presence of ERα mRNA and protein has been demonstrated in OSE and EOC in many previous studies. The present chapter elaborates on the regulation of these genes by inflammatory associated mediators, highlighting the potential regulation of oestrogen formation in OSE and EOC under the control of these locally produced immune factors. As introduced in Chapter 1, all the cytokines tested are naturally secreted in the pre- and post-ovulatory ovary and are also part of the ovarian tumour microenvironment. Moreover, mRNAs for IL-1α, IL-6 and IL-8 are expressed in primary OSE and EOC along with their cognate receptors (Ziltener, Maines-Bandiera et al. 1993; Burke, Relf et al. 1996; Merogi, Marrogi et al. 1997), suggesting intracrine, autocrine and paracrine actions thereof in OSE and ovarian cancer cells. On the other hand, IL-4 and IL-10 are not likely to be secreted by OSE, although cognate receptors are present (Burke, Relf et al. 1996), implying possible paracrine roles in the normal epithelium.

Firstly, IL-1α alone was used as a representative cytokine to test the response of these genes to inflammation in OSE and EOC. The rationale for this was firstly to minimise the number of variables in any experiment. Secondly, IL-1α is likely to exert a crucial role in ovulation induced inflammation; IL-1α activity has been identified in human follicular fluid (Barak, Yanai et al. 1992) and regulates key factors of the inflammatory cascade including PGs, NO and steroid hormone production (Gerard, Caillaud et al. 2004). Finally, IL-1α is secreted by ovarian cancer cells (Huleihel, Maymon et al. 1997). Following testing of IL-1α effects in OSE and EOC, its action on these genes in a cancer cell line, SKOV-3 was investigated, and revealed different effects of IL-1α on STS and 17βHSD5 mRNA expression.

In OSE cells, although STS and 17βHSD5 mRNA was found to be expressed, it seems that STS and 17βHSD5 mRNA is not affected by regulatory factors such as cytokines since they did not respond to 0.5ng/ml IL-1α treatment. By contrast, there was a clear decrease in EST mRNA expression in response to this IL-1α treatment and a similar pattern of expression of 17βHSD2 mRNA was seen following the same IL-1α treatment. Given the effect of EST and 17βHSD2 in the local formation of oestrogen, this decrease in mRNA expression, if translated to decreased protein expression and enzyme activity, would lead to an increase in local oestrogen availability in OSE cells. However the difference between EST and 17βHSD2 with and without IL-1α observed at the mRNA level was not repeated at the enzyme level, and the cells had equivalent enzyme activities, with no E_1 or E_2 synthesized. One explanation for this could be that Taqman quantitative analysis is a more sensitive test and is better able to highlight differences. While attempts were made to keep the experimental conditions the same for both mRNA and protein studies, there were slight differences. The density and number of cells used in each type of experiment differed, the enzyme assay used $5x10^5$ cells per well ($2x10^5$ per ml) while the mRNA studies included $3x10^5$ cells per well ($1.0\text{-}1.25x10^5$ per ml). While it is unlikely that this difference in cell number had any significant effect, there remains the possibility that the lower density of cells could influence cell response. Indeed the differences in depth of culture medium in the wells could influence gaseous exchange in both experiments. Another explanation for this could be EST enzyme activity was much stronger than that of STS in OSE, and regardless of the effect of IL-1α decreasing EST activity by more than 50%, the reduced EST activity might still be enough to counteract the STS effect, resulting in a failure to produce any E_1. Another explanation might be that the IL-1α effect on EST and 17βHSD2 transcripts was not translated into protein and consequently did not influence the enzyme activity. Since we did not have the chance to test IL-1α effect on post-menopausal OSE and OSE lining the inclusion cysts due to shortage of these cells, we do not know the exact effects of IL-1α on expression of the above genes, but we would anticipate the cells to have a similar response or even to be more sensitive to IL-1α, in which case E_1 and E_2 could probably be produced with IL-1α stimulation. However, further work

needs to be done to test the hypothesis. As far as I know this is the first report that EST and 17βHSD2 mRNA expression can be down-regulated by IL-1α in OSE cells.

Interestingly, we have found ERα to be up regulated significantly by 0.5ng/ml IL-1α in OSE cells. Although there is no E_1 and E_2 produced in OSE cells in the presence of IL-1α, there is a high concentration of E_2 in the follicular fluid over the peri-ovulatory period (Lobo, diZerega et al. 1985). OSE cells could therefore be exposed to locally high levels of E_2, which, coupled with the increased expression of ERα may exert a potent influence oestrogen-regulated genes. This adds weight to the hypothesis that inflammation may promote ovarian cancer carcinogenesis and development by regulating the oestrogen pathway. There are two previous studies on the effect of IL-1α on ER expression. One microarray analysis study on OSE cells failed to show an effect of 0.5ng/ml IL-1α for 48h on ERα mRNA expression (Rae, Niven et al. 2004b). One explanation for these conflicting results could be the different patient numbers studied. The present study tested the effect in 13 patients compared with 5 in the previous study, which would give greater credibility to the current study. In contrast, in MCF-7 breast cancer cell line Danforth reported IL-1α down-regulated ER *in vitro* (Danforth and Sgagias 1991). However, no classification of ER into ERα and ERβ subtypes was made.

Surprisingly, STS, EST, 17βHSD2, 17βHSD5 and ERα mRNA expression did not differ significantly in the presence of 0.5ng/ml IL-1α in any of the EOC samples, although there was a trend towards lower levels of EST and 17βHSD2 mRNA and a higher level of ERα mRNA in the presence of IL-1α, which is the similar to the case in OSE cells. The lack of significant differences may be due to the very small number of patient samples (n=3), and more samples should be tested before a definitive conclusion can be drawn. By contrast, in SKOV-3 cells, STS mRNA expression was observed to increase significantly with 0.15ng/ml IL-1α, and did not increase further with 0.5ng/ml. Thus 0.5ng/ml IL-1α was thought to be the optimal dose to induce STS expression. Time course studies in SKOV-3 cells showed once again a 2-fold increase in the expression of STS in response to 0.5ng/ml IL-1α from

12h to 48h. Moreover, there was a complete abrogation of the stimulatory response to IL-1α with the addition of IL-1RA. All of these experiments support the concept that IL-1α can enhance STS mRNA expression in SKOV-3 cells. In contrast to STS, the IL-1α stimulatory effect on 17βHSD5 mRNA was apparent at 12h and 24h but disappeared at 48h, and increasing the dose of IL-1α from 0.05ng/ml to 0.5ng/ml did not alter its expression at 48h. Though the effect was only transitory, it may increase the amount of 17βHSD5 protein and enzyme activity and enhance the local production of E_2. Indeed, only having a transitory increase in 17βHSD5 mRNA after IL-1α treatment might explain why there was such a modest effect of IL-1α on the ability of SKOV-3 cells to metabolize E_1S to E_2 over 72h, if the waning mRNA level was matched by falling 17βHSD5 enzyme activity. Following the mRNA studies, protein expression of the genes was assessed by functional studies of enzymatic activity in OSE and SKOV-3 cells. Only SKOV-3 cells had significantly higher enzyme levels in response to IL-1α compared to their untreated controls, which was predicted from the mRNA analysis. Like in EOC, ERα in SKOV-3 cells was not modulated by IL-1α after 48h. Studies on the regulation of oestrogen metabolizing enzymes and ER in other cancers have shown that a number of factors including cytokines can modulate the expression of these enzymes. Of these factors, the actions of interleukins have been the subject of a number of studies. Honma reported IL-6 and IL-1β enhanced proliferation of breast cancer cell lines by stimulating the activity of STS (Honma, Shimodaira et al. 2002). In contrast, Matsuoka showed that IL-1β suppressed STS mRNA and enzyme activity in endometrial stromal cells (Matsuoka, Yanaihara et al. 2002). These data suggest that the effect of cytokines on STS varies dependent on the tissue under investigation.

It is noticeable that IL-1α stimulates STS mRNA expression in SKOV-3 cells but not in EOC cells. The difference between cell lines and primary cultures may be explained by the immortal status of the cell lines, and the fact that they may have acquired further genetic mutations after many passages and long term culture, and therefore do not reflect the true *in vivo* characteristics of cancer. However, primary cancer cells are scarce, which is why cell lines were chosen in this study as an

alternative. Another explanation for this discrepancy may be that STS expression could be up regulated by IL-1α in only a small proportion of ovarian cancer patients, and the EOC cells used might be from non-responders. Nevertheless, IL-1α stimulatory effect on STS expression and activity in SKOV-3 suggests inflammation could stimulate local production of E_2 by regulating STS pathway in cancer cells from some patients.

Whilst the inflammatory cytokine IL-1α decreased EST mRNA in OSE cells, it was perhaps surprising that EST mRNA expression was also significantly decreased by the anti-inflammatory cytokine IL-4, although this is the first report demonstrating regulation of EST mRNA expression by IL-4. Down-regulation of EST mRNA and activity, leading to enhanced local oestrogen biosynthesis, might be a key component of ovarian cancer progression. There are high levels of IL-4R in OSE cells although they do not have the capacity to secrete IL-4 *de novo (*Ziltener, Maines-Bandiera et al. 1993*;* Burke, Relf et al. 1996*)*. Blood mononuclear cells that infiltrate the peri-ovulatory follicle and corpus luteum secret most of IL-4 in the pre-menopausal ovary (Hashii, Fujiwara et al. 1998). IL-4 binding to its receptors located on OSE can then inhibit the expression of EST. Given the same effect of IL-1α and IL-4 in OSE, it would be interesting to investigate if there is any synergism between these cytokines in the regulation of EST mRNA and total protein.

It was not possible to test all the cytokines on EOC cells due to limited availability, but it was possible to test their effects in SKOV-3 cells. Since there is no EST or 17βHSD2 mRNA expression in SKOV-3 cells, only the effects of cytokines on STS, 17βHSD5 and ERα expression were tested. In contrast to the varied effect of different cytokines on OSE cells, there was no effect of cytokines on STS and 17βHSD5 mRNA levels except for the stimulatory effect of IL-1α on STS mRNA expression. Moreover, IL-1α stimulates the conversion of E_1S to E_1 two fold, which indicates a two-fold increase in STS enzyme activity. It is interesting that IL-4 inhibits, while IL-8 and IL-10 stimulate ERα mRNA expression, suggesting different cytokines may have different effects on the same gene expression. Although IL-4,

IL-8 and IL-10 do not influence the expression of STS or 17βHSD5, meaning these cytokines are unable to alter the local production of active E_2, they do alter the expression of ERα providing an alternative way to affect the oestrogen responsive genes. Given the different effect of IL-4 and IL-8 or IL-10 and coexistence of these cytokines in ovarian cancer, it would be interesting to investigate if there is any synergism or antagonism between them in the regulation of ERα mRNA and total protein and thus influence the effect of E_2 in ovarian cancer.

It was noticeable that IL-1α inhibited EST mRNA expression in OSE cells (Fig. 5.1 C) while in the subsequent experiment comparing different cytokines the effect of IL-1α, although inhibitory, was not significant (Fig. 5.8 B). This can be explained by the small number of samples in the latter experiment and the variation between patient samples. Even in the former experiment, 2 of the 13 results had high EST mRNA after IL-1α treatment, but the larger number of overall samples meant the inhibitory effect of IL-1α was significant.

Because it has been demonstrated the expression of aromatase is lower than STS expression in cancer cells, the effect of inflammatory cytokines on aromatase expression and enzyme activity was not tested in the current study. However aromatase expression is stimulated by a number of factors in breast cancers. It is reported that PGE2, which is produced and secreted by breast tumor epithelial cells and fibroblasts, is the most potent factor that enhances aromatase expression via cAMP (Zhao, Agarwal et al. 1996). Additionally, it has been shown that cytokines like insulin-growth factor types I and II, IL-1, and IL-6 stimulate aromatase activity in breast tumour-derived fibroblasts in the presence of dexamethasone (Reed and Purohit 1997). Therefore, it would of interest to study the effect of cytokines on the expression of PGE2 and subsequent effect on aromatase expression and their direct effect on aromatase. Although the expression of aromatase is low, the effect of cytokines on its expression may be very dramatic, resulting in high production of E_2 and development of EOC. Thus it would provide the support for the use of anti-imflammatory and anti-aromatase treatment in EOC.

There are a few weaknesses in this part of study. Though it is better to test cytokine effects on the target genes in primary EOC cells and then compare them with OSE cells, primary EOC cells were limited. In addition, in light of the observation that different genes are maximally affected after different treatment times, it is better to perform a time course to find the optimum time for the different cytokines. However, due to limited availability of cells, and limited time and data from the result of IL-1α time course, 48h was chosen as the most suitable time point to reflect the response of the genes to different cytokines. Moreover, in previous chapters it was demonstrated that E_1S transporters like OATP-B, OATP-D and OATP-E are present in OSE and EOC cells, so it would be interesting to study their response to inflammatory cytokines.

In conclusion, Chapter 5 demonstrates that IL-1α decreases EST and 17βHSD2 and promotes ERα mRNA expression in OSE cells, suggesting a mechanism for inflammatory cytokines to promote local oestrogen biosynthesis even in the post-menopausal ovary that could favour tumourigenesis. In addition, similar to IL-1α, IL-4 decreases EST mRNA level in OSE cells. Therefore, gene transcripts of oestrogen production, metabolism and action appear to be under inflammatory control in OSE cells, and IL-1α and IL-4 are able to promote the production of E_2 in OSE cells by influencing different target genes. Moreover, IL-1α stimulates STS mRNA and activity in SKOV-3 cells. IL-4 inhibits while IL-8 and IL-10 stimulate ERα mRNA levels in SKOV-3 cells. Therefore, similar to OSE cells, gene transcripts of oestrogen production, metabolism and action are likely to be also under inflammatory control in EOC cells. Different cytokines have opposing effects, with IL-1α, IL-8 and IL-10 promoting the production or action of E_2, while IL-4 inhibits the action of E_2. Table 5.2, Table 5.3 and Table 5.4 show a summary of the data presented in this chapter.

Cytokines/genes	STS	EST	17βHSD2	17βHSD5	ERα
IL-1α	n/s	↓	↓	n/s	↑
IL-4	n/s	↓	n/s	n/s	n/s
IL-6	n/s	n/s	n/s	n/s	n/s
IL-8	n/s	n/s	n/s	n/s	n/s
IL-10	n/s	n/s	n/s	n/s	n/s

Table 5.2: Summary of cytokine effects on STS, EST, 17βHSD2, 17βHSD5 and ERα mRNA in OSE cells. n/s: non-significant; ↑ : up-regulation; ↓ : down-regulation.

Cytokines/genes	STS	EST	17βHSD2	17βHSD5	ERα
IL-1α	n/s	n/s	n/s	n/s	n/s
IL-4	n/s	n/s	n/s	n/s	n/s
IL-6	n/s	n/s	n/s	n/s	n/s
IL-8	n/s	n/s	n/s	n/s	n/s
IL-10	n/s	n/s	n/s	n/s	n/s

Table 5.3: Summary of cytokine effects on STS, EST, 17βHSD2, 17βHSD5 and ERα mRNA in EOC cells. n/s: non-significant.

Cytokines/genes	STS	EST	17βHSD2	17βHSD5	ERα
IL-1α	↑	-	-	n/s	n/s
IL-4	n/s	-	-	n/s	↓
IL-6	n/s	-	-	n/s	n/s
IL-8	n/s	-	-	n/s	↑
IL-10	n/s	-	-	n/s	↑

Table 5.4: Summary of cytokine effects on STS, 17βHSD5 and ERα mRNA in SKOV-3 cells.

n/s: non-significant; ↑ : up-regulation; ↓ : down-regulation; - : no expression .

Chapter 6

Oestrogenic regulation of cancer-associated cellular activity in ovarian cancer cell line

6.1 Introduction

As demonstrated in previous chapters, genes encoding the production and metabolism of oestrogens are present in ovarian cancer and active E_2 could be produced by EOC cells through the STS pathway. Moreover, oestrogen receptors are expressed in EOC cells. It is therefore reasonable to ask the question: what is the effect of E_2 in EOC cells? There are many scientific and clinical studies concerning different aspects of the effect oestrogen in ovarian cancer. Firstly, there is potential role of oestrogen in the initiation of ovarian cancer. One animal study demonstrated that E_2 can induce ovarian serous cysts, and diethylstilbestrol (DES) can induce surface papillary neoplasm in guinea pigs (Silva, Tornos et al. 1998). Another study in mice revealed E_2 accelerates the initiation of ovarian cancer resulting in decreased survival time in a transgenic mouse model (Laviolette, Garson et al. 2010). However, the molecular mechanisms by which oestrogen could give rise to cancer are poorly understood. Secondly, there is the role of oestrogen in the development of cancer. E_2 increases the growth rate of PEO-4, an ER positive EOC cell line and this stimulation can be blocked by tamoxifen (Langdon, Hawkes et al. 1990). Moreover, E_2 can lead to clear morphological changes characteristic of epithelial-mesenchymal transition and enhance ovarian cancer cell migratory ability (Park, Cheung et al. 2008). However, the role of oestrogen is controversial. Rochefort showed oestrogen inhibited invasion and motility of ovarian cancer cells (Rochefort, Platet et al. 1998). Therefore the precise effect of oestrogen on the development of ovarian cancer remains to be clarified. Last but not least, some clinical trials complement these studies of effect of oestrogen on ovarian cancer. In a phase II study of letrozole, an AI, it was demonstrated that ER-positive ovarian cancer patients had no disease progression following 6 months of treatment (Smyth, Gourley et al. 2007).

As discussed in Chapter 1, HRT including ERT has a longstanding tradition in the treatment of menopausal complaints and has benefited many women. However, alongside the benefits of HRT, there are some potential risks, such as breast cancer, endometrial cancer and venous thromboembolism. More and more epidemiological studies demonstrate higher ovarian cancer risks with ERT users, but there are few

studies on the mechanisms of this higher risk. There are many HRT regimes which rely on oral administration of CEEs and E_2, which are the first two most widely used oral oestrogens. Premarin, commercial CEEs tablets, contains at least 10 oestrogens, the most abundant of which are E_1S (48%) and EqS (26%) (Whittaker, Morgan et al. 1980). Therefore to find the potential mechanism behind the higher risk of ovarian cancer with ERT users, Eq and EqS were selected as the exogenous oestrogen sources to treat the cells.

In this chapter, it is hypothesised that exogenous oestrogens from the HRT can be converted to active oestrogen via STS pathway by EOC cells and cause the same alteration of cancer-associated genes as endogenous oestrogen, thereby assisting the development of EOC. In order to test this hypothesis, PEO-1, an ER-positive and ER-responsive EOC cell line, was chosen as an EOC model. The effect of E_2, Eq and their sulphated products, E_1S, EqS on the migration of the cells was tested. Moreover, effect of E_2, Eq and E_1S, EqS on four cancer-associated genes, IGFBP3, FN1, LOX and E-cadherin was investigated. Anti-oestrogen and anti-STS treatments were also included to confirm the action of oestrogen and STS.

6.2 Materials and methods

6.2.1 Cell line

PEO-1 cell line was from Dr Simon Langdon (Cancer Research UK, Edinburgh Oncology Unit, Edinburgh, UK).

6.2.2 *In vitro* wound healing assay

The wound healing assay was performed as described in Section 2.6.

6.2.3 RNA extraction and reverse transcription

RNA was extracted using methods described in Section 2.4.1 and RT-PCR was performed using standard conditions as described in Section 2.4.2.

6.2.4 Taqman qRT-PCR

QRT-PCR was performed using a 7900 sequence detection system and analysed as described in Section 2.4.3. The probes and primers used were commercially available pre-validated from Applied Biosystems Assay on Demand or designed in house. The information of the primers and probes are shown in Table 2.3.

6.2.5 Statistical analysis

Statistical analysis was performed using GraphPad Prism 5. Data from wound healing assays and qRT-PCR (except Sections 6.3.2.3 and 6.3.3.3) were analyzed by one-way ANOVA with Tukey post-hoc test. QRT-PCR data in Sections 6.3.2.3 and 6.3.3.3 were analyzed by two-way ANOVA. Statistical difference was assigned at $P<0.05$.

6.3 Results

6.3.1 Effect of oestrogen on cell migration

6.3.1.1 Effect of E_2 and Eq on cell migration

Treatment of PEO-1 cells with 10^{-8}M E_2 and Eq *in vitro* for 48h resulted in stimulation of cell migration measured as wound closure, although this effect was not statistically significant (Fig. 6.1, A). Addition of ICI 182,780 (10^{-6}M) tended to reverse this effect (Fig. 6.1, B).

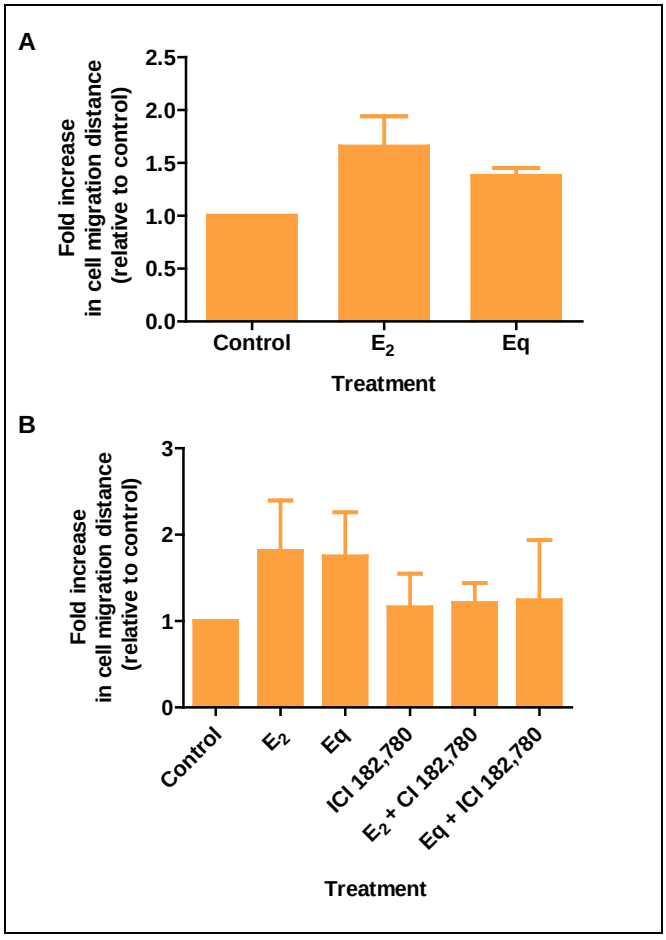

Figure 6.1: Effect of E_2, Eq and ICI 182,780 on PEO-1 cell migration. Cells were cultured for 48h with E_2 (10^{-8}M), Eq (10^{-8}M) (A, B) or +/- ICI 182,780 (10^{-6}M) (B) and wound closure measured as the distance of cell migration was determined using a microscope. Bars indicate Mean±SEM. (n=3)

6.3.1.2 Effect of E_1S and EqS on cell migration

Treatment of PEO-1 cells with 10^{-8}M E_1S *in vitro* for 72h resulted in a two-fold increase in wound closure and addition of the STS inhibitor, STX64, (10^{-5}M, added twice at 0h and 48h respectively) significantly reversed this effect (Fig. 6.2, A, P<0.01). Treatment of PEO-1 cells with 10^{-8}M NaEqS *in vitro* for 72h resulted in a non-significant increase in wound closure but addition of STX64 (10^{-5}M) did not significantly reduce wound closure (Fig. 6.2, B).

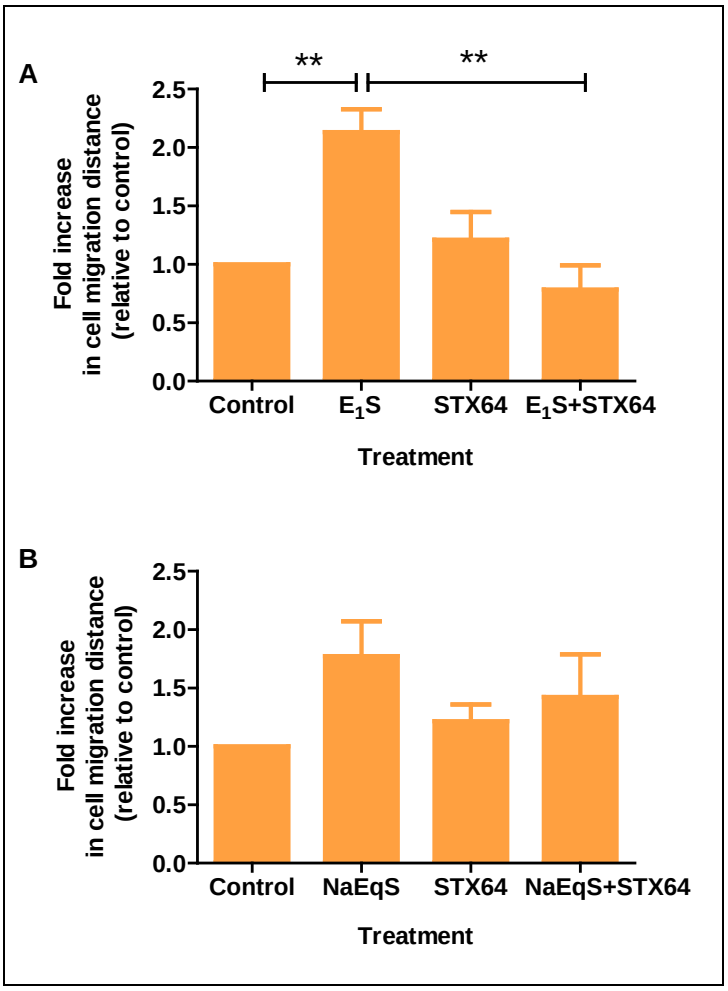

Figure 6.2: Effect of E_1S, NaEqS and STX64 on PEO-1 cell migration. Cells were cultured with E_1S (10^{-8}M) +/- STX (10^{-5}M) (A) and NaEqS (10^{-8}M-) +/- STX64 (10^{-5}M) (B) for 72h and wound closure measured as the distance of cell migration was determined using a microscope. Bars indicate Mean±SEM. (n=3, **=P<0.01)

6.3.2 Effect of oestrogen on FN1 mRNA expression in PEO-1 cells

6.3.2.1 Effect of E_2 and Eq on FN1 mRNA expression

PEO-1 cells were treated with 10^{-8}M E_2 or Eq *in vitro* for 48h. FN1 mRNA expression was significantly reduced by approximately 50% in the presence of either steroid (Fig. 6.3, P<0.05).

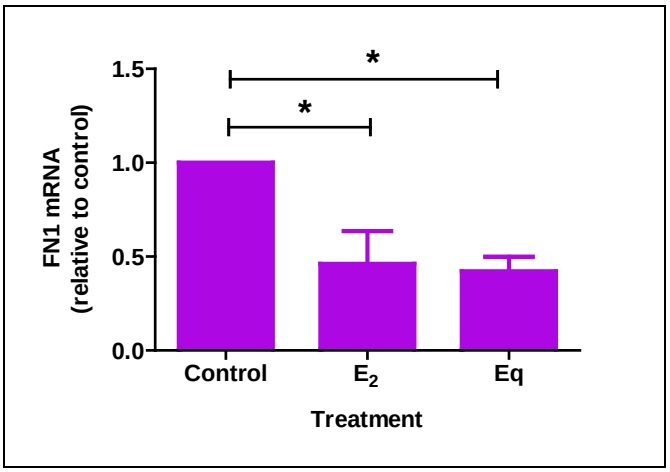

Figure 6.3: Effect of E_2 and Eq on FN1 mRNA expression in PEO-1 cells. PEO-1 cells were treated with E_2 (10^{-8}M) or Eq (10^{-8}M) for 48h and expression of FN1 mRNA was measured by Taqman qRT-PCR. Bars indicate Mean±SEM. Asterisks indicate significant difference from untreated control. (n=3, *=P<0.05)

6.3.2.2 Dose-related effect of E_2 and Eq on FN1 mRNA expression

PEO-1 cells were treated with increasing doses of E_2 (10^{-12}M, 10^{-10}M and 10^{-8}M) *in vitro* for 48h. FN1 mRNA expression was inhibited significantly by E_2 (10^{-10} and 10^{-8}M) and the strongest suppression was with 10^{-10}M E_2 (Fig. 6.4, A, P<0.05, P<0.01). PEO-1 cells were also treated with increasing doses of Eq (10^{-12}M, 10^{-10}M and 10^{-8}M) *in vitro* for 48h. FN1 mRNA expression was suppressed significantly by all doses of Eq and the strongest inhibition was with 10^{-10}M Eq (Fig. 6.4, B, P<0.001).

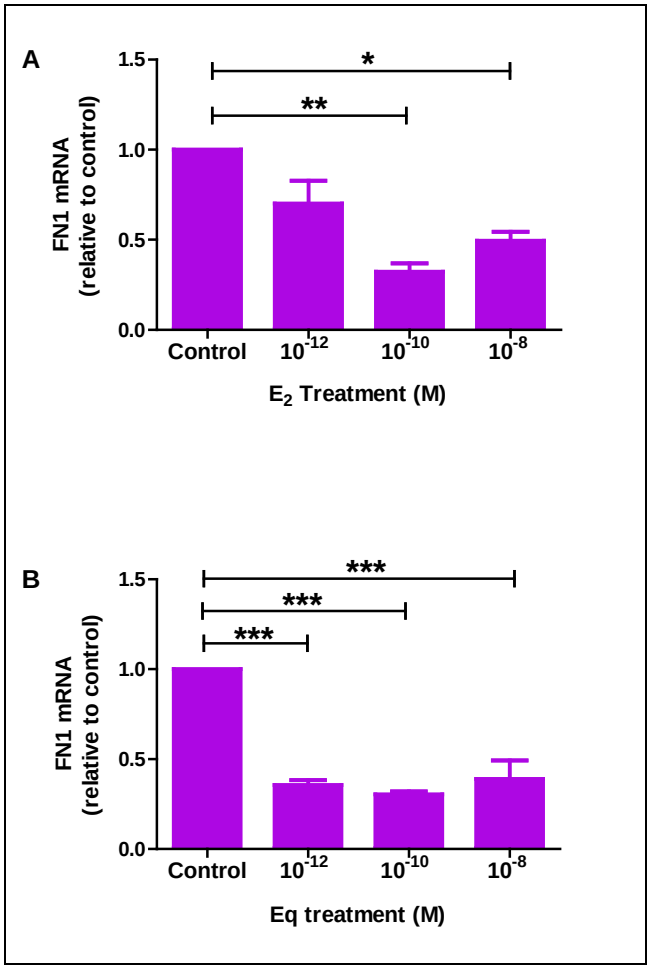

Figure 6.4: Dose-related effect of E_2 and Eq on FN1 mRNA expression in PEO-1 cells. PEO-1 cells were treated with increasing doses of E_2 (A) and Eq (B) (10^{-12}M-10^{-8}M) for 48h and expression of FN1 mRNA was measured by Taqman qRT-PCR. Bars indicate Mean±SEM. Asterisks indicate significant difference from untreated control. (n=3, *=P<0.05, **=P<0.01, ***=P<0.001)

6.3.2.3 Time-dependent effect of E_2 on FN1 mRNA expression

As shown above, treatment of PEO-1 cells with increasing doses of E_2 for 48h significantly inhibited FN1 mRNA. When a fixed dose of E_2 (10^{-10}M) was added to PEO-1 cells E_2 inhibited FN1 mRNA in a time-dependent manner. Maximum inhibition (2-fold) was measured after 24h (Fig. 6.5, P<0.01).

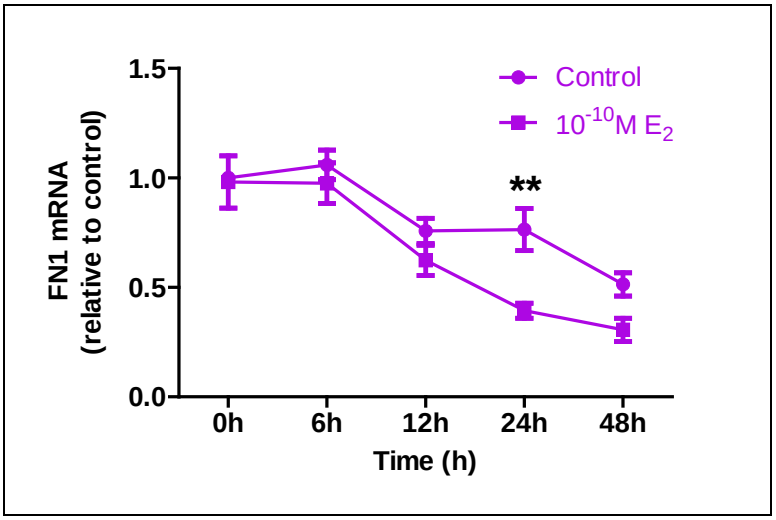

Figure 6.5: Time-dependent effect of E_2 on FN1 mRNA expression in PEO-1 cells. E_2 treatment (10^{-8}M) was applied in a time dependent manner (6, 12, 24, 48h). Expression of FN1 mRNA was measured by Taqman qRT-PCR. Bars indicate Mean±SEM. Asterisks indicate significant difference from untreated control at the same time point. (n=3, **=P<0.01)

6.3.2.4 Effect of the ER antagonist, ICI 182,780 on E_2 and Eq inhibition of FN1 mRNA expression

Treatment of PEO-1 cells with 10^{-8}M E_2 and Eq *in vitro* for 48h resulted in significant inhibition of FN1 mRNA expression, as previously observed (Fig. 6.3). Addition of ICI 182,780 (10^{-6}M) reversed E_2 and Eq inhibitory effect (Fig. 6.6, P<0.01, P<0.001).

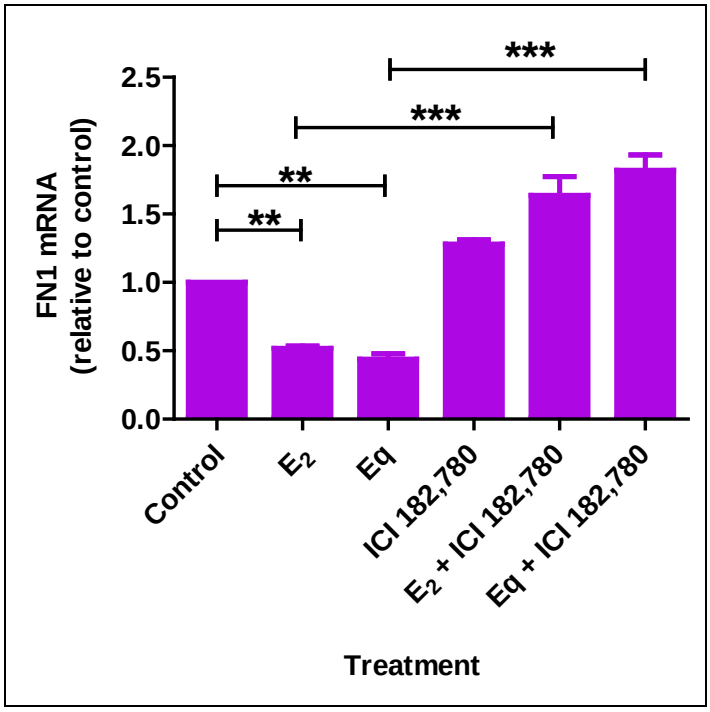

Figure 6.6: FN1 mRNA expression in PEO-1 cells treated with E_2, Eq and ICI 182,780. PEO-1 cells were treated with E_2 (10^{-8}M) or Eq (10^{-8}M) or +/- ICI 182,780 (10^{-6}M) for 48h and expression of FN1 mRNA was measured with Taqman qRT-PCR. Bars indicate Mean±SEM. (n=3, **=P<0.01, ***=P<0.001)

6.3.2.5 Dose-related effect of E_1S and EqS on FN1 mRNA expression

PEO-1 cells were treated with increasing doses of E_1S and NaEqS (10^{-10}M, 10^{-8}M and 10^{-6}M) *in vitro* for 72h. FN1 mRNA expression was inhibited in a dose-dependent manner by both E_1S and NaEqS the strongest suppression was with 10^{-6}M E_1S (Fig. 6.7, A, B, P<0.001).

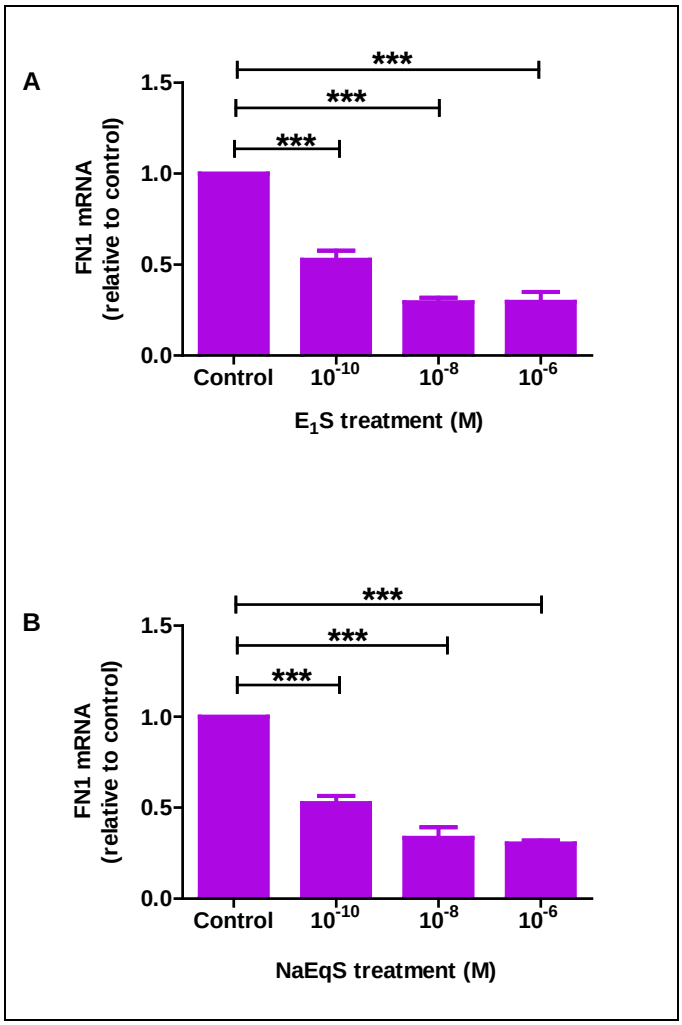

Figure 6.7: Dose response of E_1S and NaEqS on FN1 mRNA expression in PEO-1 cells. PEO-1 cells were treated with increasing doses of E_1S (A) and NaEqS (B) (10^{-10}M-10^{-6}M) for 72h and expression of FN1 mRNA was measured with Taqman qRT-PCR. Bars indicate Mean±SEM. Asterisks indicate significant difference from untreated control. (n=3, ***=P<0.001)

6.3.2.6 Effect of the STS inhibitor, STX64, on E_1S and NaEqS inhibition of FN1 mRNA expression

As observed previously (Fig. 6.7, A) treatment of PEO-1 cells with increasing doses of E_1S (10^{-10}M, 10^{-8}M and 10^{-6}M) for 72h resulted in dose-dependent suppression of FN1 mRNA expression, and the inhibitory effect of E_1S (10^{-8}M and 10^{-6}M) was statistically significant. Addition of STX64 (10^{-5}M) reversed the inhibitory effect of E_1S (10^{-8}M and 10^{-6}M) and this was significant at E_1S (10^{-6}M) (Fig. 6.8, A, P<0.01, P<0.001). Similarly, increasing dose of NaEqS (10^{-10}M, 10^{-8}M and 10^{-6}M) tended to inhibit FN1 mRNA expression and STX64 (10^{-5}M) tended to reverse the inhibitory effect although the differences were not statistically significant (Fig. 6.8, B).

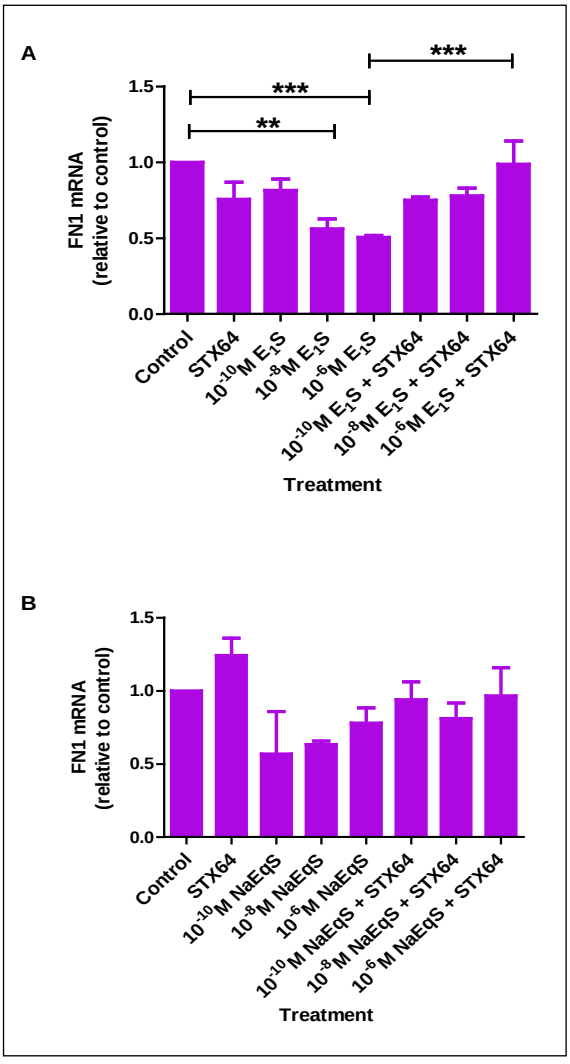

Figure 6.8: FN1 mRNA expression in PEO-1 cells treated with E_1S, NaEqS and STX64. PEO-1 cells were treated with increasing dose of E_1S (A), NaEqS (B) (10^{-10}M-10^{-6}M) or +/- STX64 (10^{-5}M) for 72h and expression of FN1 mRNA was measured with Taqman qRT-PCR. Bars indicate Mean±SEM. (n=3, **=P<0.01, ***=P<0.001)

6.3.3 Effect of oestrogen on IGFBP3 mRNA expression in PEO-1 cells

6.3.3.1 Effect of E_2 and Eq on IGFBP3 mRNA expression

PEO-1 cells were treated with 10^{-8}M E_2 or Eq *in vitro* for 48h. IGFBP3 mRNA expression was significantly inhibited by approximately 60% (Fig. 6.9, P<0.001).

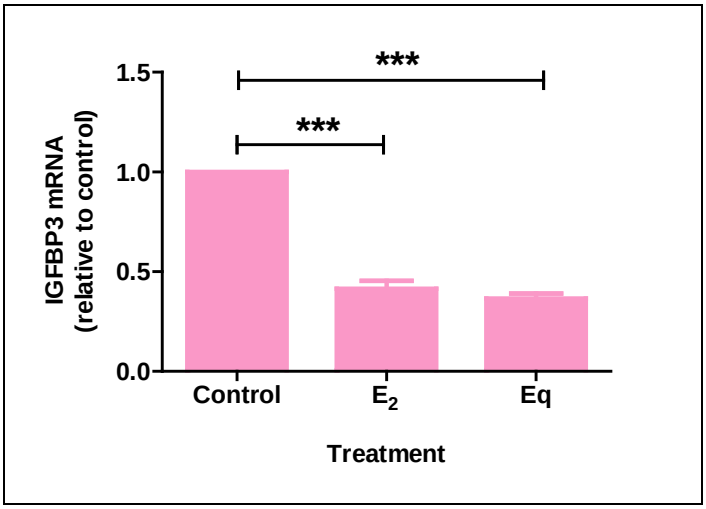

Figure 6.9: Effect of E_2 and Eq on IGFBP3 mRNA expression in PEO-1 cells. PEO-1 cells were treated with E_2 (10^{-8}M) or Eq (10^{-8}M) for 48h and expression of IGFBP3 mRNA was measured with Taqman qRT-PCR. Bars indicate Mean±SEM. Asterisks indicate significant difference from untreated control. (n=3, ***=P<0.001)

6.3.3.2 Dose-related effect of E_2 and Eq on IGFBP3 mRNA expression

PEO-1 cells were treated with increasing doses of E_2 (10^{-12}M, 10^{-10}M and 10^{-8}M) *in vitro* for 48h. IGFBP3 mRNA expression was inhibited in a dose-dependent manner by E_2 and the strongest suppression was with 10^{-8}M E_2 (Fig. 6.10, A, P<0.05, P<0.001). PEO-1 cells were also treated with increasing doses of Eq (10^{-12}M, 10^{-10}M and 10^{-8}M) *in vitro* for 48h. IGFBP3 mRNA expression was suppressed significantly by Eq, although the effect was not dose-dependent and the strongest inhibition was with 10^{-12}M Eq (Fig. 6.10, B, P<0.05).

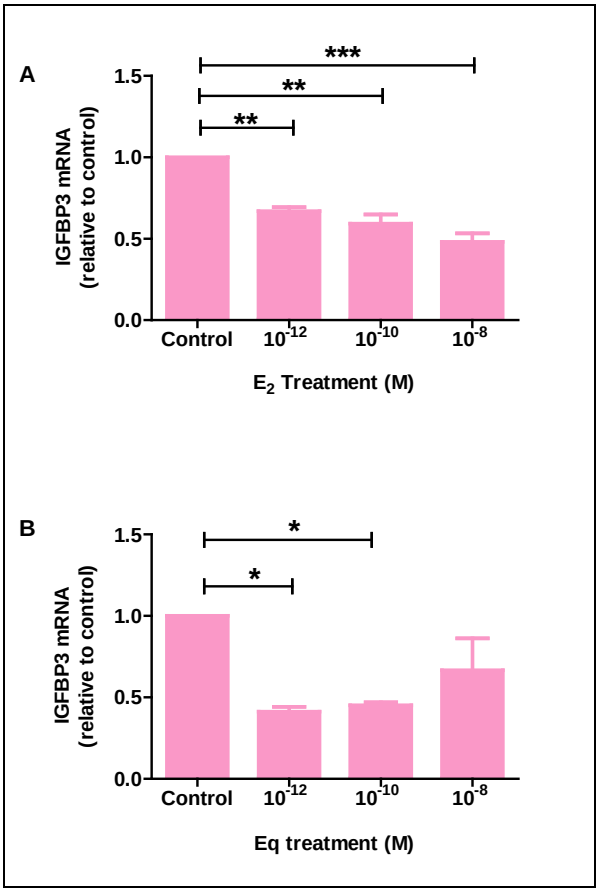

Figure 6.10: Dose-related effect of E_2 and Eq on IGFBP3 mRNA expression in PEO-1 cells. PEO-1 cells were treated with increasing dose of E_2 (A) and Eq (B) (10^{-12}M-10^{-8}M) for 48h and expression of IGFBP3 mRNA was measured with Taqman qRT-PCR. Bars indicate Mean±SEM. Asterisks indicate significant difference from untreated control. (n=3, *=P<0.05, **=P<0.01, ***=P<0.001)

6.3.3.3 Time dependent effect of E_2 on FN1 mRNA expression

As shown above, treatment of PEO-1 cells with increasing doses of E_2 for 48h significantly inhibited IGFBP3 mRNA. When a fixed dose of E_2 (10^{-10}M) was added to PEO-1 cells, a significant 2-fold supression of IGFBP3 mRNA was observed after 6h, 24h and 48h (Fig. 6.11, P<0.01, P<0.001).

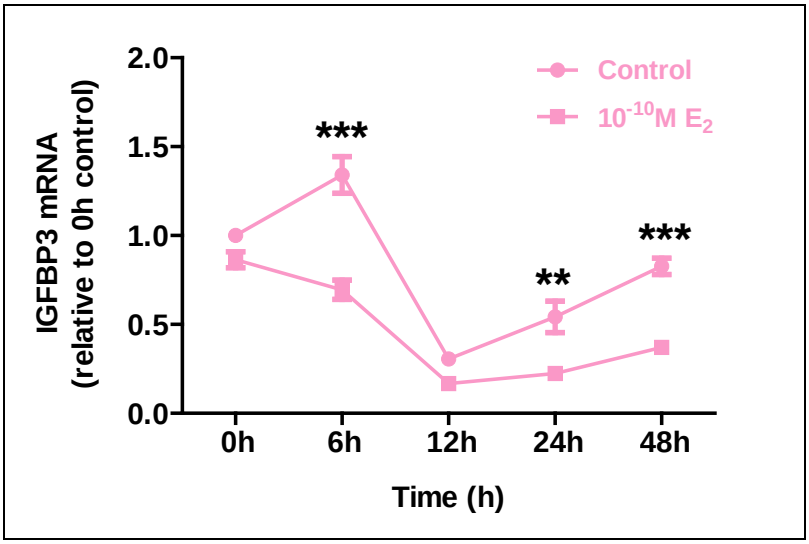

Figure 6.11: Time-dependent effect of E_2 on IGFBP3 mRNA expression in PEO-1 cells. Expression of IGFBP3 mRNA was measured 0, 6, 12, 24 and 48h after addition of E_2 (10^{-8}M) by Taqman qRT-PCR. Bars indicate Mean±SEM. Asterisks indicate significant difference from untreated control at the same time point. (n=3, **=P<0.01, ***=P<0.001)

6.3.3.4 Effect of the ER antagonist, ICI 182,780 on E_2 and Eq inhibition of IGFBP3 mRNA expression

Treatment of PEO-1 cells with 10^{-8}M E_2 and Eq *in vitro* for 48h resulted in significant inhibition of IGFBP3 mRNA expression. ICI 182,780 (10^{-6}M) alone suppressed IGFBP3 mRNA expression significantly. Addition of ICI 182,780 did not reverse E_2 and Eq inhibitory effect (Fig. 6.12, P<0.01, P<0.001).

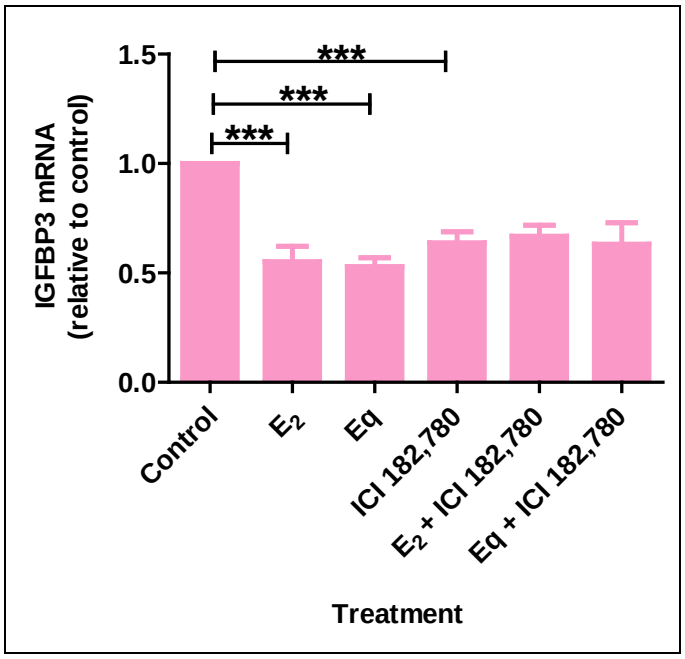

Figure 6.12: IGFBP3 mRNA expression in PEO-1 cells treated with E_2, Eq and ICI 182,780. PEO-1 cells were treated with E_2 (10^{-8}M) or Eq (10^{-8}M) or +/- ICI 182,780 (10^{-6}M) for 48h and expression of IGFBP3 mRNA was measured with Taqman qRT-PCR. Bars indicate Mean±SEM. (n=3, **=P<0.01, ***=P<0.001)

6.3.3.5 Dose-related effect of E_1S and EqS on IGFBP3 mRNA expression

PEO-1 cells were treated with increasing doses of E_1S (10^{-10}M, 10^{-8}M and 10^{-6}M) *in vitro* for 72h. IGFBP3 mRNA expression was inhibited significantly by E_1S and the strongest suppression was with 10^{-8}M E_1S (Fig. 6.13, A, P<0.05, P<0.001). PEO-1 cells were also treated with increasing doses of NaEqS (10^{-10}M, 10^{-8}M and 10^{-6}M) *in vitro* for 72h. IGFBP3 mRNA expression was suppressed significantly by NaEqS and the strongest inhibition was with 10^{-8}M NaEqS (Fig. 6.13, B, P<0.01, P<0.001). In both cases the response to treatment with the sulphated steroid resulted in suppression that approximated a dose-dependent effect.

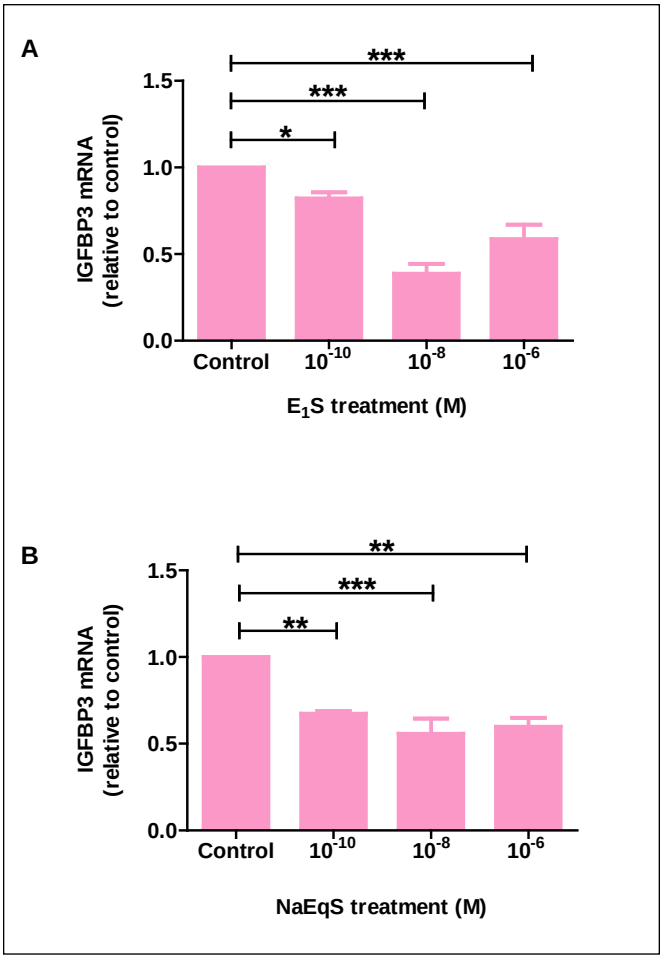

Figure 6.13: Dose response of E_1S and NaEqS on IGFBP3 mRNA expression in PEO-1 cells. PEO-1 cells were treated with increasing dose of E_1S (A) and NaEqS (B) (10^{-10}M-10^{-6}M) for 72h and expression of IGFBP3 mRNA was measured with Taqman qRT-PCR. Bars indicate Mean±SEM. Asterisks indicate significant difference from untreated control. (n=3, *=P<0.05, **=P<0.01, ***=P<0.001)

6.3.3.6 Effect of the STS inhibitor, STX64, on E_1S and NaEqS inhibition of IGFBP3 mRNA expression

Treatment of PEO-1 cells with increasing doses of E_1S (10^{-10}M, 10^{-8}M and 10^{-6}M) for 72h resulted in suppression of IGFBP3 mRNA expression in a dose-dependent manner (compare with Fig. 6.13A) and the inhibitory effect of E_1S at 10^{-8}M and 10^{-6}M was statistically significant. STX64 (10^{-5}M) alone suppressed IGFBP3 mRNA expression and addition of STX64 did not reverse the inhibitory effect of E_1S (Fig. 6.14, A, $P<0.001$). Similarly, NaEqS (10^{-10}M, 10^{-8}M and 10^{-6}M) inhibited IGFBP3 mRNA expression in a dose-dependent manner (compare with Fig. 6.13B) and STX64 (10^{-5}M) alone suppressed IGFBP3 mRNA expression and addition of STX64 did not reverse inhibitory effect of E_1S (Fig. 6.14, B, $P<0.05$, $P<0.01$, $P<0.001$).

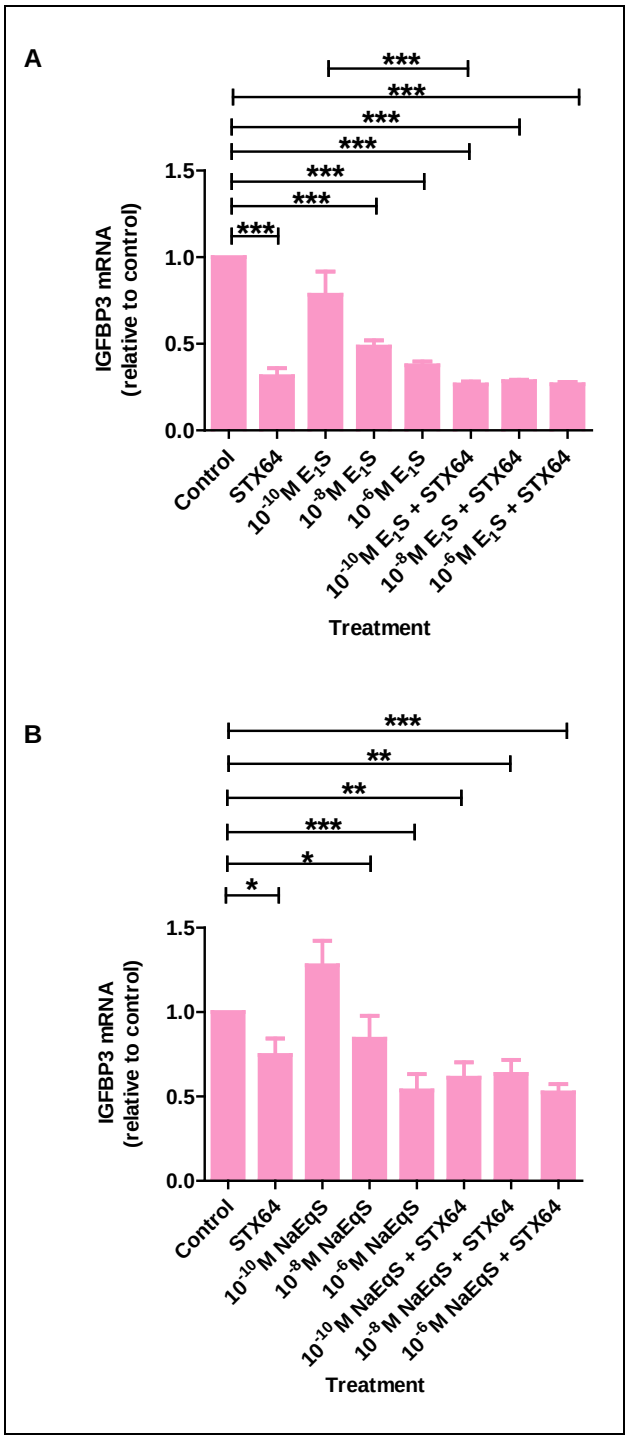

Figure 6.14: IGFBP3 mRNA expression in PEO-1 cells treated with E_1S, NaEqS and STX64. PEO-1 cells were treated with increasing dose of E_1S (A), NaEqS (B) (10^{-10}M-10^{-6}M) or +/- STX64 (10^{-5}M) for 72h and expression of IGFBP3 mRNA was measured with Taqman qRT-PCR. Bars indicate Mean±SEM. (n=3, *=P<0.05, **=P<0.01, ***=P<0.001)

6.3.4 Effect of oestrogen on LOX mRNA expression in PEO-1 cells

PEO-1 cells were treated with 10^{-8}M E_2 or Eq *in vitro* for 48h. There was no effect of E_2 or Eq on LOX mRNA expression (Fig. 6.15).

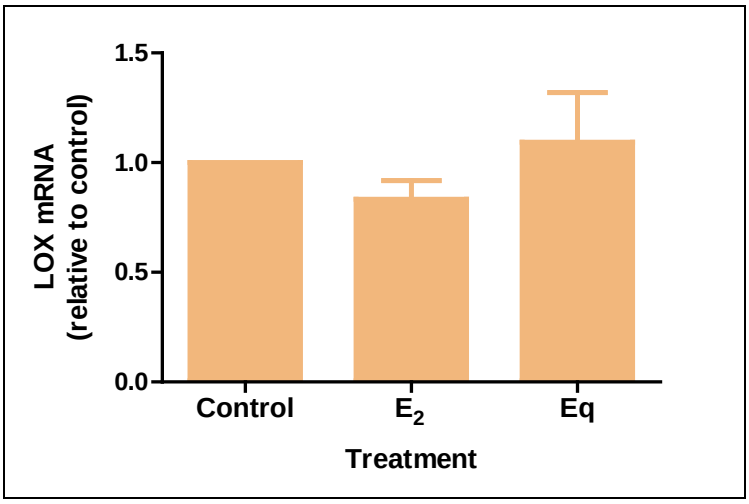

Figure 6.15: Lack of effect of E_2 and Eq on LOX mRNA expression in PEO-1 cells. PEO-1 cells were treated with E_2 (10^{-8}M) or Eq (10^{-8}M) for 48h and expression of LOX mRNA was measured with Taqman qRT-PCR. Bars indicate Mean±SEM. (n=3)

6.3.5 Effect of oestrogen on E-cadherin mRNA expression in PEO-1 cells

PEO-1 cells were treated with 10^{-8}M E_2 or Eq *in vitro* for 48h. There was no effect of E_2 or Eq on E-cadherin mRNA expression (Fig. 6.16).

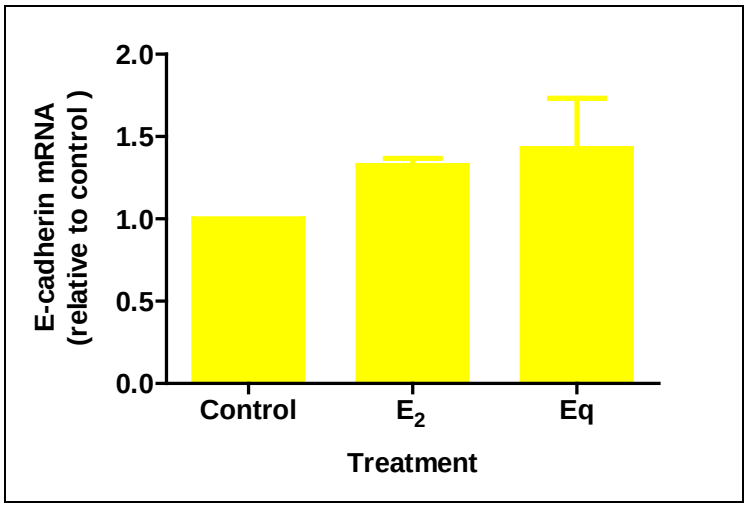

Figure 6.16: Lack of effect of E_2 and Eq on E-cadherin mRNA expression in PEO-1 cells. PEO-1 cells were treated with E_2 (10^{-8}M) or Eq (10^{-8}M) for 48h and expression of E-cadherin mRNA was measured with Taqman qRT-PCR. Bars indicate Mean±SEM. (n=3)

6.4 Discussion

Despite research on ovarian cancer over many years there have been no significant improvements in clinical outcomes. Thus identification of important factors that can influence EOC development is often regarded with enthusiasm and expectation. Although oestrogens as potential tumour promoting factors for EOC have excited international scientific interest, the mechanism of oestrogen action in the process is still not clear. A few epidemiological data have indicated that HRT users have a higher risk of ovarian cancer, while there are few studies on the mechanism of this effect. The data presented in this chapter are the first to demonstrate possible pathways by which oestrogens derived from CEE, a popular oestrogen form of HRT, might influence the development of EOC and provide potential methods to prevent and treat this disease.

As demonstrated in Chapter 4 there is high ERα mRNA expression in the PEO-1 cell line and growth of these cells is stimulated by E_2 implying the presence of functional ER (O'Donnell, Macleod et al. 2005). Therefore PEO-1 is regarded as a good oestrogen-responsive EOC model. In the present chapter, the effect of E_2 and Eq on the migration of PEO-1 cells was tested first. As expected, the migration of the cells was stimulated by both E_2 and Eq and the effect could be reversed by ER (ERα and ERβ) antagonist ICI 182,780, although this was not statistically significant, suggesting that further repeats of this experiment are required to reach statistical significance. If proven to be correct, this would indicate that Eq, one of CEE metabolites, has the same effect as E_2 in promoting the migration of PEO-1 cells. Similarly, E_1S and EqS stimulated the migration of the cells. In the case of E_1S this was significant, and the effect could be blocked by STX64, an STS inhibitor. This suggests that E_1S and EqS, components of CEEs, were converted to active oestrogens, E_2 and Eq, through the action of STS in the cancer cells, with the active oestrogen products acting on the cells to increase their migration. Although there is no published data on the effect of STX64 on the migration of cancer cells, there are studies about its inhibitory effect on the proliferation of cancer cells and growth of tumours. STX64 inhibited the proliferation of MCF-7 cells (breast cancer cell line)

overexpressing STS cDNA (MCF-7_{STS}) by 32% and caused about 20% MCF-7_{STS} tumour regression (Foster, Chander et al. 2008b). In addition, STX64 inhibited the growth of Ishikawa (endometrial cancer cell line) xenograft in ovariectomized mice by 48% when1mg/kg STX64 was given to the mice daily (Foster, Chander et al. 2008a). Measurement of the width of the wounds in the present study was done manually, meaning it was relatively subjective, although this should be compensated to some extent by measuring 3 points on every scratch and obtaining the average width. Further studies should be done to repeat these experiments or include another method to measure the migration of the cells to reduce the error. Moreover, another functional test, such as a cell proliferation assay to study the effect of oestrogen on the proliferation of the cancer cells could be included.

Having demonstrated that both natural and CEE-derived oestrogens have similar effects on promoting cell migration, selected E_2-responsive cancer-associated genes in PEO-1 cells were then measured to test the effect of E_2 and Eq. A number of E_2-regulated genes have been identified in PEO-1 cells by gene microarray. Among these genes, IGFBP3 and FN1 have the two largest fold-changes that were confirmed by real-time RT-PCR (O'Donnell, Macleod et al. 2005). FN1 and IGFBP3 are reported to be involved in the development of cancers. IGFBP3 has been shown to act as potent inhibitor of IGF-IR signaling in several non-small cell lung cancer cell lines, causing growth arrest and inducing apoptosis (Lee, Chun et al. 2002). Recombinant IGFBP3 inhibited basal and E_2-stimulated MCF-7 breast cancer cell proliferation (Huynh, Yang et al. 1996). In ovarian cancer, IGFBP3 has been associated with disease stage and residual tumour volume when measured by ELISA or by quantitative RT-PCR (Katsaros, Yu et al. 2001; Lu, Katsaros et al. 2006; Wiley, Katsaros et al. 2006). FN1 is an ECM glycoprotein, which is involved in cell adhesion, migration, growth and differentiation. Its expression was found to be down regulated in ovarian cancer and was related to histological grade (Chang, Zhang et al. 2009). The reduction in FN1 and IGFBP3 mRNA in response to E_2 was consistent with the O'Donnell study, although the fold change was smaller in the present study. Moreover, time-course studies of E_2 on FN1 and IGFBP3 mRNA expression confirmed the inhibitory effect of E_2 on these two genes in PEO-1 cells. Therefore

the reduced expression of FN1 and IGFBP3 by E_2 can influence apoptosis, proliferation and migration of cancer cells. As expected, similar to the effect of E_2, Eq treatment dramatically decreased FN1 and IGFBP3 mRNA expression in PEO-1 cells, and the effect was further confirmed by the observation that FN1 and IGFBP3 mRNA expression was inhibited by Eq in a dose-dependent manner. Together, the above results suggest both E_2 and Eq can influence the development of EOC by regulating cancer-associated genes. The ER antagonist ICI 182,780 abrogated the E_2 inhibitory effect on FN1 mRNA expression. Surprisingly, ICI 182,780 alone decreased IGFBP3 mRNA expression and did not reverse E_2 inhibitory effect on IGFBP3 mRNA expression. The mechanism behind this effect requires further study but it is possible that ICI 182,780 activates other unknown pathways while blocking ER. Agonistic effects of ICI 182,780 have also been found in some other cells. Robertson found agonistic up-regulation of GAPDH mRNA in superficial endometrial cells and antagonistic down-regulation of ER and PR mRNAs in the inner layer of the myometrium of the sheep uterus, and concluded that the agonist versus antagonist effects of ICI 182,780 relative to those of E_2 were a function of the gene examined, as well as the specific cell within the uterus (Robertson, Zhang et al. 2001). Moreover, Pinto concluded that ICI 182,780 has agonistic effects on several typical oestrogenic responses in fish, but its actions are tissue-specific. They observed ICI 182,780 mimicked some E_2 actions on gene expression in sea bream liver, up regulating ER alpha, vitellogenin II and choriogenin L, but did not have these effects in the testis (Pinto, Singh et al. 2006). The agonistic effect of ICI 182,780 suggests caution in the use of ICI 182,780 as a pure anti-oestrogen to block oestrogen function, and may explain why there are some EOC cases insensitive to anti-oestrogen treatments.

As mentioned above, although E_2 has an inhibitory effect on FN1 and IGFBP3 mRNA in the present study, the effect is not as clear cut as described in O'Donnell's study (O'Donnell, Macleod et al. 2005). The difference in fold-change inhibition might be explained by the different passage numbers of the PEO-1 cells. The present study used the cells with higher passage numbers in which the sensitivity to oestrogen treatments might be reduced.

As confirmation of the ability of PEO-1 to metabolize sulphated oestrogens, and indicative of the presence of STS and 17βHSD1/5, FN1 and IGFBP3 mRNAs expression was significantly suppressed by 10^{-8}M and 10^{-6}M E_1S. Similarly NaEqS inhibited IGFBP3 mRNA expression significantly and tended to reduce FN1 mRNA expression, although there was no statistical significance, suggesting the conversion of NaEqS to Eq and subsequent regulation of the cancer-associated genes. STX64 was used to confirm whether E_1S and NaEqS were converted by the STS pathway. As predicted, STX64 abrogated the E_1S inhibitory effect on FN1 mRNA expression significantly and a suggestion of reversion of E_1S inhibitory effect on FN1 mRNA, although this was not significant. Surprisingly, STX64 alone decreased IGFBP3 mRNA expression and did not reverse E_1S and NaEqS inhibitory effect on IGFBP3 mRNA expression. Interestingly, this effect of STX64 is similar to ICI 182,780 since both decreased IGFBP3 mRNA but not FN1 mRNA. The mechanism behind this effect requires further study but it is possible that STX64 actives other unknown pathways while blocking the STS pathway. Both the E_1S and NaEqS inhibitory effects indicate that the components of CEEs can regulate the oestrogen-responsive cancer-associated genes, and then influence the development of EOC.

As introduced in Chapter 1, STX64 is a tricylic coumarin-based sulfamate that irreversibly inhibits STS activity. It was selected for the first Phase I clinical trial of an STS inhibitor in postmenopausal women with breast cancer. As expected it was able to almost completely block STS activity in peripheral blood lymphocytes and tumour tissues. Serum concentrations of E_1, E_2, androstenediol and DHEA all decreased significantly from pre-treatment levels. Therefore STX64 is shown to be an efficient and well-tolerated STS inhibitor for breast cancer treatment (Stanway, Purohit et al. 2006). Since the present results show there is active STS converting inactive E_1S to E_1 and then to active E_2 in EOC, STX64 was evaluated to test its inhibitory effect on STS action in EOC. Its different effects on FN1 and IGFBP3 mRNA expression suggest different regulatory mechanisms on different genes. Although it can inhibit STS function and thus abrogate E_2 effects on some cancer-associated genes it may also activate other pathways to influence other genes. To

determine its overall effect on EOC, STX64 effect on the proliferation and migration of EOC cells treated with E_2 precursors need to be further investigated.

Due to increased female longevity, women now spend nearly half of their adult lives in a state of relative oestrogen deficiency. HRT consisting of daily oral administration of CEEs either alone or in combination with MPA are effective at relieving menopausal symptoms. However there are many absolute contraindications to HRT, such as cancer of the breast, cancer of the endometrium, endometrioid ovarian cancer, thromboembolic disorders amongst others (Kubba 1995). Ovarian cancer other than endometrioid ovarian cancer is not included in the contraindications. The finding in this study that CEE-derived oestrogen can influence cancer-associated genes in EOC raises caution concerning the clinical use of the oestrogen-containing HRT. It is worthwhile evaluating whether ovarian cancer or family history of ovarian cancer should be included in the absolute contraindications. If individual patients with EOC do require HRT treatment, the physician should think about the forms and duration of HRT carefully, since local application of oestrogen does not influence the serum concentration of oestrogen and short-term use has less opportunity of causing and promoting EOC.

As discussed in Chapter 1, both LOX and E-cadherin are expressed in EOC cells and are known to be involved in the development of many cancers (Birchmeier and Behrens 1994; Ren, Yang et al. 1998; Erler, Bennewith et al. 2006). Therefore, in addition to IGFBP3 and FN1, the effect of E_2 and Eq on these two genes was also tested. However, there was no effect of E_2 or Eq on LOX and E-cadherin. Thus E_2 and Eq only regulate specific cancer-associated genes and it is important to identify these specific genes since enhancement or blockade of the relevant pathways may provide novel potential treatments for EOC.

In this chapter only one EOC cell line was chosen to test the hypothesis, while primary EOC cells would have been the ideal choice. However, each patient has their own characteristics, so responses to E_2 in different primary cancer cells are likely to

be variable. It would take considerable time to identify consistent oestrogen-responsive primary cells. Given the limited time and resources, an EOC cell line was used. The discoveries of stimulatory effect of E_2 on the migration of EOC cell and inhibitory effect on cancer-associated genes will inform further research on primary EOC cells.

In summary, Chapter 6 demonstrated that both E_2 and Eq, metabolites of CEEs by EOC cells, and E_1S and NaEqS, ingredients of CEEs, tend to stimulate the migration of PEO-1 cells. Moreover, both E_2 and Eq dramatically decrease FN1 and IGFBP3 mRNA expression in PEO-1 cell line. ICI 182,780, an ER antagonist, abrogates the E_2 inhibitory effect on FN1 mRNA expression while alone it decreases IGFBP3 mRNA expression. In addition, both E_1S and NaEqS inhibit FN1 and IGFBP3 mRNA expression. STX64, an STS inhibitor, abrogates the E_1S inhibitory effect on FN1 mRNA expression while alone it decreases IGFBP3 mRNA expression. These results indicate that local metabolism of inactive oestrogen components of HRT can influence the development of ovarian cancer by regulating cancer-associated genes. Thus we demonstrate a potential mechanism for HRT-associated oestrogen-mediated enhancement of ovarian cancer development.

Chapter 7

General discussion and future directions

7.1 General discussion

This thesis explored oestrogen pre-receptor synthesis and metabolism as well as action in human OSE and EOC. This was performed by studying expression patterns of oestrogen producing and metabolizing enzymes and ERs using IHC, Taqman qRT-PCR and enzyme activity assay. Additionally, inflammatory regulation of the enzymes and ERs by different cytokines was investigated *in vitro*. Furthermore, the action of HRT-derived oestrogen on oestrogen-responsive cancer-associated genes was examined in an EOC cell line.

The first hypothesis of the thesis is that oestrogen producing and metabolizing enzymes exist in EOC, facilitating local E_2 production. In Chapters 3 and 4, the results established that the proteins required for local oestrogen production and metabolism such as STS, EST, 17βHSD2 and 17βHSD5 were localized in pre-menopausal, post-menopausal and inclusion cystic OSE as well as EOC cells. STS, EST, 17βHSD1, 17βHSD2, 17βHSD5, ERα, ERβ, OATP-B, OATP-D and OATP-E mRNAs were also differentially expressed in pre-menopausal OSE and EOC. EST mRNA expression was significantly higher in OSE compared to EOC cells while OATP-B mRNA expression was the reverse. Radiometric enzyme activity assays demonstrated different patterns of E_1S and E_1 metabolism between OSE and EOC cells. The balance of overall activities of STS and 17βHSD1 or 17βHSD5 were higher than the overall activities of EST and 17βHSD2 in cancer cells favouring E_1 and E_2 production from E_1S in EOC, but the reverse was true in OSE cells, favouring E_2 inactivation from E_1S. This suggests OSE tends to be protected from the formation of active oestrogen while EOC tends to promote the production of active oestrogen.

The second hypothesis of the thesis is that inflammatory cytokines regulate the development of EOC by promoting the synthesis of oestrogen in EOC. In Chapter 5, the data revealed that in OSE cells, EST and 17βHSD2 mRNA was decreased while ERα mRNA was increased by IL-1α. Together, these changes in gene expression in response to IL-1α would tend to promote active oestrogen synthesis in OSE and suggest a mechanism for inflammatory promotion of tumourigenesis in OSE by local

up regulation of active oestrogen biosynthesis. In addition, EST mRNA was inhibited by IL-4. In SKOV-3 cells, IL-1α stimulated STS mRNA and enzyme activity, resulting in increased conversion of E_1S to E_1, again favouring active oestrogen formation. Moreover, IL-4 inhibited while IL-8 and IL-10 enhanced ERα mRNA expression. Together, these results suggest oestrogen production via STS/EST pathways in OSE and EOC is regulated by a number of inflammatory cytokines, although different genes are regulated by different cytokines in OSE compared with EOC cells. Moreover, in addition to the regulation at the pre-receptor level, oestrogen action is also be regulated at receptor level with different effects of cytokines on ERα mRNA expression in OSE and EOC.

The third hypothesis of the thesis is that exogenous CEEs can be converted to active oestrogens locally in EOC, playing an important role in the development of EOC by regulating oestrogen-responsive genes. In Chapter 6, the results demonstrated mRNA expression of two cancer-associated genes, FN1 and IGFBP3, which were inhibited by both E_2 and Eq as well as E_1S and NaEqS. ICI 182,780 (ER antagonist) and STX64 (STS enzyme inhibitor) abrogate the E_2, Eq, E_1S, and NaEqS inhibitory effect on FN1 mRNA expression, while alone they decrease IGFBP3 mRNA expression. In contrast to FN1 and IGFBP3, another two cancer-associated oestrogen-related genes, LOX and E-cadherin, did not respond to E_2 and Eq. These results suggest exogenous CEEs used in HRT can be converted to active oestrogens locally by EOC, causing altered expression of specific cancer-associated genes, which participate in the proliferation or migration of the cancer cells, similar to the effect of endogenous E_2. This finding provides an insight into the cancer-promoting effects of HRT, which contain CEEs, and justifies further experimental and clinical research on the application of anti-oestrogen and anti-STS compounds in the treatment of EOC.

In conclusion this thesis presents evidence that oestrogen pre-receptor production and metabolism occurs in OSE and EOC cells, and E_2 formation is inhibited in normal OSE but is promoted in EOC. Inflammatory cytokines also influence the local production of E_2 by regulating oestrogen metabolic genes and receptors. Finally, local HRT metabolites can regulate cancer-associated gene expression in

EOC. Together, these data suggest a mechanism for local oestrogen production and action that can promote tumour development in EOC, and OSE cells from pre-menopausal women, whilst protected from these effects in the quiescent ovary, are likely exposed to higher active oestrogen in an inflammatory environment.

7.2 Future studies and clinical implications

The studies presented in this thesis have shown the expression of oestrogen pre-receptor metabolizing enzymes and active E_2 produced in EOC based on a small number of EOC samples. In Chapter 3, seven EOC tissues were tested by IHC and in chapter 4 mRNA levels in four EOC patients were measured by Taqman qRT-PCR. These results have provided initial evidence from limited numbers of patients. Further experiments with more patients would strengthen this observation. Moreover, since there are 4 main subtypes of EOC, it would be of interest to measure STS/EST enzyme expression in different subtypes of EOC. The main advantage of the classification is to help identify the subtypes that are more sensitive to oestrogen stimulation, thus helping select the patients most suitable for anti-oestrogen treatment. Furthermore, studying the association of genes such as STS, EST, 17βHSD2, 17βHSD5, ERα, OATP-B, OATP-D and OATP-E with the risk of EOC recurrence and survival time, would be helpful in determining which genes or combination of genes can be used as independent factors for prognosis. Based on the above discussion an experiment could be designed using a tissue microarray to test the expression of above proteins on 50 serous, mucinous, endometrioid and clear cell EOC respectively, and compare to EOC recurrence and survival time.

As demonstrated in this thesis, STS and 17βHSD5 play an important role in conversion of inactive E_1S to active E_2 in EOC, and E_2 can influence the development of EOC by regulating cancer-associated genes. Thus, blocking STS pathway in EOC might be a potential treatment for EOC. In Chapter 6 the STS inhibitor, STX64, was shown to significantly reduce E_2 stimulatory effect on the migration of PEO-1 cells and reverse E_2 inhibitory effect on FN1, but not on IGFBP3 mRNA expression. This is strong evidence to support the use of STX64 as a

treatment for EOC, although further experiments need to be performed. *In vitro* experiments such as testing E_2 effects on the proliferation or invasion of the cancer cells should be included. Moreover, *in vivo* experiments using a xenograft model would provide further supporting evidence. PEO-1 cells can be injected into the flanks of nude mice to produce EOC tumours. Mice could then be given oral STX64 to test its effect on reducing tumour growth. Once such pre-clinical models have demonstrated STX64 effectiveness in suppressing the growth of EOC *in vivo*, a stage I clinical trial could be designed and applied to investigate STX64 efficiency of treating EOC. Recently, a 2nd generation of STS inhibitor, STX213, has been developed. It has been tested pre-clinically and is shown to be more potent and have a longer duration of action *in vivo* in both rats and mice compared to STX64 (Foster, Chander et al. 2008b). It would therefore be interesting to test STX213 effect on treating EOC and compare it with STX64. Another approach to blocking STS function would be RNA interference (RNAi). It would be interesting to test STS expression and activity after RNAi treatment in EOC cells. Since 17βHSD5 is responsible for the final step in the biosynthesis of E_2 from E_1S in EOC, inhibition of its function alone or together with STS would be potentially more effective. Further work testing the effect of a 17βHSD5 inhibitor or knocking down expression of 17βHSD5 by RNAi would be of interest.

The results in Chapter 4 showed lower expression of EST mRNA in EOC compared with OSE cells, and consequent interconversion between E_1 and E_1S in opposite directions. Since EST opposes the effect of STS, increasing the expression and activity of EST combined with inhibition of STS could be an approach to the treatment of EOC. Further investigation should include both *in vitro* and *in vivo* experiments. Firstly, the expression of EST and metabolism of E_1S in EOC could be retested after EST was transfected to EOC cells. Secondly, the proliferation, migration and invasion of EOC cells could also be compared after transfection of EST. If the transfection was effective and enhanced expression of EST, thereby reducing the biosynthesis and action of E_2, *in vivo* experiments on a xenograft model as described above could be designed and tested.

The main focus of this thesis is on the STS pathway of oestrogen synthesis, since the mRNA and protein expression of aromatase is much lower than STS. However, this does not exclude the possible production of E_2 through aromatisation. As introduced in Chapter 1, after menopause, the serum concentration of DHEAS is much higher than that of E_1S. STS and 3βHSD1 in EOC could then convert DHEAS to A, an aromatase substrate. If aromatase is expressed in some EOC patients, although the expression is low, since the concentration of precursor A is higher, the production of E_2 might be significant. Allowing for conversion from DHEAS there is potentially a high concentration of A in the serum after menopause, which could diffuse into EOC and be converted to E_2 by aromatase. Therefore it would be worth testing aromatase expression in a large number of EOC samples to find out if there is a subgroup of EOC with higher expression of aromatase alone or together with STS. In addition, dynamic conversion of exogenous A or T to E_2 (aromatase enzyme activity) could be tested in short term cultures of EOC cells. Aromatase inhibitors (AIs) could be given to the patients with higher aromatase expression, or recently-developed dual aromatase-sulphatase inhibitors (DASIs) which can block both STS and aromatase could be tried on the patients with higher expression of both enzymes.

Studies on STS pathway and STS inhibitors extend the repertoire of possible anti-oestrogen treatments for EOC. However, this raises the question of how to choose the most suitable anti-oestrogen treatment clinically: namely anti-STS, anti-aromatase or anti-ER. The relative expression of STS, aromatase or ER might be one criterion, and subtypes of EOC, age, stage might be other subsidiary criteria. Further clinical research is required to find the most effective treatment.

As stated in Chapter 1, although OSE is usually regarded as the cell of origin of EOC, other compelling evidence has suggested distal tubal epithelium is another possible precursor of some types of EOC. Therefore separation and culture of primary distal tubal epithelium cells, investigation of production and action of oestrogen in these cells and comparison with OSE and EOC cells would be useful to clarify the effect of oestrogen on initiation and promotion of different kinds of EOC. There is increasing evidence that ovarian cancers contain cancer stem-like cells which might be

responsible for the tumourigenesis and chemo-resistance of ovarian cancer (Bapat, Mali et al. 2005). Moreover, oestrogen has been shown to expand breast cancer stem-like cells through paracrine FGF/Tbx3 signalling (Fillmore, Gupta et al. 2010). The role of oestrogen in the development of EOC is studied in the thesis but the influence of oestrogen on the stem cell population has not been assessed. It would be of much interest to isolate the ovarian cancer stem-like cells, investigate their biosynthesis of oestrogen, and test the effect of oestrogen on the differentiation, proliferation and apoptosis of these cells.

Oestrogen metabolism pathways in OSE and EOC are studied in detail in this thesis, and are shown to play an important role in the development of EOC, while there are many other hormones including GnRH, gonadotrophins, androgen and progesterone which also have recognized effects on OSE and EOC. Although there are experimental studies investigating the effect of these hormones on EOC separately, their action together has not been studied. Clinically, GnRH agonist, anti-androgen and progesterone have been used in refractory or recurrent ovarian cancer patients who have failed one or more chemotherapeutic treatments, and are effective in some cases. However, combinations of these hormonal therapies have not been used consistently. Therefore, it is potentially promising to explore the effect of different hormone combinations in EOC treatment, such as oestrogen together with anti-androgen, GnRH antagonist or progesterone agonist, which would provide evidence to support their combined use in clinical practice in the future.

In the thesis, the expression of target genes was compared between pre-menopausal OSE and EOC cells. We imagine that post-menopausal OSE cells would be more suitable for the comparison, since most EOC occur after menopause. However a shortage of postmenopausal OSE prevented extended exploration. Therefore it would be important to establish a proper post-menopausal OSE cell line model. This kind of cell line would provide further opportunity to study the biology of postmenopausal OSE and initiation of EOC.

Bibliography

Aberle, H., H. Schwartz, et al. (1996). "Cadherin-catenin complex: protein interactions and their implications for cadherin function." J Cell Biochem **61**(4): 514-23.

Abisogun, A. O., D. Daphna-Iken, et al. (1988). "Modulatory role of eicosanoids in vascular changes during the preovulatory period in the rat." Biol Reprod **38**(4): 756-62.

Adami, H. O., C. C. Hsieh, et al. (1994). "Parity, age at first childbirth, and risk of ovarian cancer." Lancet **344**(8932): 1250-4.

Akahira, J., T. Suzuki, et al. (2002). "Differential expression of progesterone receptor isoforms A and B in the normal ovary, and in benign, borderline, and malignant ovarian tumors." Jpn J Cancer Res **93**(7): 807-15.

Akhmedkhanov, A., P. Toniolo, et al. (2001). "Aspirin and epithelial ovarian cancer." Prev Med **33**(6): 682-7.

Al Sarakbi, W., R. Mokbel, et al. (2006). "The role of STS and OATP-B mRNA expression in predicting the clinical outcome in human breast cancer." Anticancer Res **26**(6C): 4985-90.

Altinoz, M. A. and R. Korkmaz (2004). "NF-kappaB, macrophage migration inhibitory factor and cyclooxygenase-inhibitions as likely mechanisms behind the acetaminophen- and NSAID-prevention of the ovarian cancer." Neoplasma **51**(4): 239-47.

Ames, B. N., L. S. Gold, et al. (1995). "The causes and prevention of cancer." Proc Natl Acad Sci U S A **92**(12): 5258-65.

Arend, W. P. and C. J. Guthridge (2000). "Biological role of interleukin 1 receptor antagonist isoforms." Ann Rheum Dis **59 Suppl 1**: i60-4.

Asadullah, K., W. Sterry, et al. (2003). "Interleukin-10 therapy--review of a new approach." Pharmacol Rev **55**(2): 241-69.

Auersperg, N., S. L. Maines-Bandiera, et al. (1997). "Ovarian carcinogenesis and the biology of ovarian surface epithelium." J Cell Physiol **173**(2): 261-5.

Auersperg, N., A. S. Wong, et al. (2001). "Ovarian surface epithelium: biology, endocrinology, and pathology." Endocr Rev **22**(2): 255-88.

Balen, A. (1995). "The effects of ovulation induction with gonadotrophins on the ovary and uterus and implications for assisted reproduction." Hum Reprod **10**(9): 2233-7.

Balkwill, F. and A. Mantovani (2001). "Inflammation and cancer: back to Virchow?" Lancet **357**(9255): 539-45.

Bapat, S. A., A. M. Mali, et al. (2005). "Stem and progenitor-like cells contribute to the aggressive behavior of human epithelial ovarian cancer." Cancer Res **65**(8): 3025-9.

Barak, V., N. Mordel, et al. (1992). "The correlation of interleukin 1 and tumour necrosis factor to oestradiol, progesterone and testosterone levels in periovulatory follicular fluid of in-vitro fertilization patients." Hum Reprod **7**(4): 462-4.

Barak, V., P. Yanai, et al. (1992). "Interleukin-1: local production and modulation of human granulosa luteal cells steroidogenesis." Fertil Steril **58**(4): 719-25.

Bardin, A., P. Hoffmann, et al. (2004). "Involvement of estrogen receptor beta in ovarian carcinogenesis." Cancer Res **64**(16): 5861-9.

Bardin, A., F. Moll, et al. (2005). "Transcriptional and posttranscriptional regulation of fibulin-1 by estrogens leads to differential induction of messenger ribonucleic acid variants in ovarian and breast cancer cells." Endocrinology **146**(2): 760-8.

Baserga, R. (1996). "Controlling IGF-receptor function: a possible strategy for tumor therapy." Trends Biotechnol **14**(5): 150-2.

Beral, V., E. Banks, et al. (1997). "Hormone replacement therapy and high incidence of breast cancer between mammographic screens." Lancet **349**(9058): 1103-4.

Beral, V., D. Bull, et al. (2007). "Ovarian cancer and hormone replacement therapy in the Million Women Study." Lancet **369**(9574): 1703-10.

Bhavnani, B. R. (2003). "Estrogens and menopause: pharmacology of conjugated equine estrogens and their potential role in the prevention of neurodegenerative diseases such as Alzheimer's." J Steroid Biochem Mol Biol **85**(2-5): 473-82.

Birchmeier, W. and J. Behrens (1994). "Cadherin expression in carcinomas: role in the formation of cell junctions and the prevention of invasiveness." Biochim Biophys Acta **1198**(1): 11-26.

Blomquist, C. H., M. Bonenfant, et al. (2002). "Androgenic and estrogenic 17beta-hydroxysteroid dehydrogenase/17-ketosteroid reductase in human ovarian epithelial tumors: evidence for the type 1, 2 and 5 isoforms." J Steroid Biochem Mol Biol **81**(4-5): 343-51.

Bowman, A., H. Gabra, et al. (2002). "CA125 response is associated with estrogen receptor expression in a phase II trial of letrozole in ovarian cancer: identification of an endocrine-sensitive subgroup." Clin Cancer Res **8**(7): 2233-9.

Brandenberger, A. W., M. K. Tee, et al. (1998). "Estrogen receptor alpha (ER-alpha) and beta (ER-beta) mRNAs in normal ovary, ovarian serous cystadenocarcinoma and ovarian cancer cell lines: down-regulation of ER-beta in neoplastic tissues." J Clin Endocrinol Metab **83**(3): 1025-8.

Brannstrom, M., N. Bonello, et al. (1995). "Effects of tumour necrosis factor alpha (TNF alpha) on ovulation in the rat ovary." Reprod Fertil Dev **7**(1): 67-73.

Brannstrom, M. and R. J. Norman (1993). "Involvement of leukocytes and cytokines in the ovulatory process and corpus luteum function." Hum Reprod **8**(10): 1762-75.

Brannstrom, M., L. Wang, et al. (1993). "Ovulatory effect of interleukin-1 beta on the perfused rat ovary." Endocrinology **132**(1): 399-404.

Brinton, L. A., G. Gridley, et al. (1997). "Cancer risk after a hospital discharge diagnosis of endometriosis." Am J Obstet Gynecol **176**(3): 572-9.

Brown, P. O. and C. Palmer (2009). "The preclinical natural history of serous ovarian cancer: defining the target for early detection." PLoS Med **6**(7): e1000114.

Bruning, P. F., J. Van Doorn, et al. (1995). "Insulin-like growth-factor-binding protein 3 is decreased in early-stage operable pre-menopausal breast cancer." Int J Cancer **62**(3): 266-70.

Burger, H. G. and S. R. Davis (2002). "The role of androgen therapy." Best Pract Res Clin Obstet Gynaecol **16**(3): 383-93.

Burke, F., M. Relf, et al. (1996). "A cytokine profile of normal and malignant ovary." Cytokine **8**(7): 578-85.

Buscher, U., F. C. Chen, et al. (1999). "Cytokines in the follicular fluid of stimulated and non-stimulated human ovaries; is ovulation a suppressed inflammatory reaction?" Hum Reprod **14**(1): 162-6.

Byrns, M. C. and T. M. Penning (2009). "Type 5 17beta-hydroxysteroid dehydrogenase/prostaglandin F synthase (AKR1C3): role in breast cancer and inhibition by non-steroidal anti-inflammatory drug analogs." Chem Biol Interact **178**(1-3): 221-7.

Cannistra, S. A. (1993). "Cancer of the ovary." N Engl J Med **329**(21): 1550-9.

Cardillo, M. R., E. Petrangeli, et al. (1998). "Androgen receptors in ovarian tumors: correlation with oestrogen and progesterone receptors in an immunohistochemical and semiquantitative image analysis study." J Exp Clin Cancer Res **17**(2): 231-7.

Casey, M. L. and P. C. MacDonald (1997). "Lysyl oxidase (ras recision gene) expression in human amnion: ontogeny and cellular localization." J Clin Endocrinol Metab **82**(1): 167-72.

Chadha, S., B. R. Rao, et al. (1993). "An immunohistochemical evaluation of androgen and progesterone receptors in ovarian tumors." Hum Pathol **24**(1): 90-5.

Chang, X. H., L. Zhang, et al. (2009). "Screening for genes associated with ovarian cancer prognosis." Chin Med J (Engl) **122**(10): 1167-72.

Chang, Y. S., G. Kong, et al. (2002a). "Clinical significance of insulin-like growth factor-binding protein-3 expression in stage I non-small cell lung cancer." Clin Cancer Res **8**(12): 3796-802.

Chang, Y. S., L. Wang, et al. (2002b). "Correlation between insulin-like growth factor-binding protein-3 promoter methylation and prognosis of patients with stage I non-small cell lung cancer." Clin Cancer Res **8**(12): 3669-75.

Chen, L. L., F. Ye, et al. (2009). "Evaluation of immune inhibitory cytokine profiles in epithelial ovarian carcinoma." J Obstet Gynaecol Res **35**(2): 212-8.

Chen, V. W., B. Ruiz, et al. (2003). "Pathology and classification of ovarian tumors." Cancer **97**(10 Suppl): 2631-42.

Chien, C. H., F. F. Wang, et al. (1994). "Transcriptional activation of c-myc proto-oncogene by estrogen in human ovarian cancer cells." Mol Cell Endocrinol **99**(1): 11-9.

Choi, K. C., S. K. Kang, et al. (2002). "Follicle-stimulating hormone activates mitogen-activated protein kinase in preneoplastic and neoplastic ovarian surface epithelial cells." J Clin Endocrinol Metab **87**(5): 2245-53.

Chura, J. C., C. H. Blomquist, et al. (2009). "Estrone sulfatase activity in patients with advanced ovarian cancer." Gynecol Oncol **112**(1): 205-9.

Clinton, G. M., C. Rougeot, et al. (1996). "Estrogens increase the expression of fibulin-1, an extracellular matrix protein secreted by human ovarian cancer cells." Proc Natl Acad Sci U S A **93**(1): 316-20.

Coenen, C. M., C. M. Thomas, et al. (1996). "Changes in androgens during treatment with four low-dose contraceptives." Contraception **53**(3): 171-6.

Colette, S., J. C. Lousse, et al. (2009). "Absence of aromatase protein and mRNA expression in endometriosis." Hum Reprod **24**(9): 2133-41.

Colotta, F., F. Re, et al. (1993). "Interleukin-1 type II receptor: a decoy target for IL-1 that is regulated by IL-4." Science **261**(5120): 472-5.

Cottreau, C. M., R. B. Ness, et al. (2003). "Endometriosis and its treatment with danazol or lupron in relation to ovarian cancer." Clin Cancer Res **9**(14): 5142-4.

Coughlin, S. S., A. Giustozzi, et al. (2000). "A meta-analysis of estrogen replacement therapy and risk of epithelial ovarian cancer." J Clin Epidemiol **53**(4): 367-75.

Csiszar, K., S. F. Fong, et al. (2002). "Somatic mutations of the lysyl oxidase gene on chromosome 5q23.1 in colorectal tumors." Int J Cancer **97**(5): 636-42.

Cunat, S., F. Rabenoelina, et al. (2005). "Aromatase expression in ovarian epithelial cancers." J Steroid Biochem Mol Biol **93**(1): 15-24.

Danforth, D. N., Jr. and M. K. Sgagias (1991). "Interleukin 1 alpha blocks estradiol-stimulated growth and down-regulates the estrogen receptor in MCF-7 breast cancer cells in vitro." Cancer Res **51**(5): 1488-93.

Darai, E., R. Detchev, et al. (2003). "Serum and cyst fluid levels of interleukin (IL) -6, IL-8 and tumour necrosis factor-alpha in women with endometriomas and benign and malignant cystic ovarian tumours." Hum Reprod **18**(8): 1681-5.

Darai, E., J. Y. Scoazec, et al. (1997). "Expression of cadherins in benign, borderline, and malignant ovarian epithelial tumors: a clinicopathologic study of 60 cases." Hum Pathol **28**(8): 922-8.

de los Santos, M. J., D. J. Anderson, et al. (1998). "Expression of interleukin-1 system genes in human gametes." Biol Reprod **59**(6): 1419-24.

del Carmen, M. G., A. F. Fuller, et al. (2003). "Phase II trial of anastrozole in women with asymptomatic mullerian cancer." Gynecol Oncol **91**(3): 596-602.

Dibbelt, L., R. Knuppen, et al. (1991). "Group comparison of serum ethinyl estradiol, SHBG and CBG levels in 83 women using two low-dose combination oral contraceptives for three months." Contraception **43**(1): 1-21.

Ding, J., D. Li, et al. (2008). "Fibronectin promotes invasiveness and focal adhesion kinase tyrosine phosphorylation of human colon cancer cell." Hepatogastroenterology **55**(88): 2072-6.

Dower, S. K., S. R. Kronheim, et al. (1986). "The cell surface receptors for interleukin-1 alpha and interleukin-1 beta are identical." Nature **324**(6094): 266-8.

Dreher, D. and A. F. Junod (1996). "Role of oxygen free radicals in cancer development." Eur J Cancer **32A**(1): 30-8.

Drolet, R., M. Simard, et al. (2007). "Human type 2 17 beta-hydroxysteroid dehydrogenase mRNA and protein distribution in placental villi at mid and term pregnancy." Reprod Biol Endocrinol **5**: 30.

Dufort, I., P. Rheault, et al. (1999). "Characteristics of a highly labile human type 5 17beta-hydroxysteroid dehydrogenase." Endocrinology **140**(2): 568-74.

Edmondson, R. J., J. M. Monaghan, et al. (2002). "The human ovarian surface epithelium is an androgen responsive tissue." Br J Cancer **86**(6): 879-85.

Emons, G., C. Grundker, et al. (2003). "GnRH antagonists in the treatment of gynecological and breast cancers." Endocr Relat Cancer **10**(2): 291-9.

Enmark, E., M. Pelto-Huikko, et al. (1997). "Human estrogen receptor beta-gene structure, chromosomal localization, and expression pattern." J Clin Endocrinol Metab **82**(12): 4258-65.

Erler, J. T., K. L. Bennewith, et al. (2006). "Lysyl oxidase is essential for hypoxia-induced metastasis." Nature **440**(7088): 1222-6.

Espey, L. L. (1980). "Ovulation as an inflammatory reaction--a hypothesis." Biol Reprod **22**(1): 73-106.

Espey, L. L. (1994). "Current status of the hypothesis that mammalian ovulation is comparable to an inflammatory reaction." Biol Reprod **50**(2): 233-8.

Espey, L. L. (1999). "Ovulation." Encyclopedia of Reproduction **3**: 605-614.

Evangelou, A., S. K. Jindal, et al. (2000). "Down-regulation of transforming growth factor beta receptors by androgen in ovarian cancer cells." Cancer Res **60**(4): 929-35.

Fathalla, M. F. (1971). "Incessant ovulation--a factor in ovarian neoplasia?" Lancet **2**(7716): 163.

ffrench-Constant, C. (1995). "Alternative splicing of fibronectin--many different proteins but few different functions." Exp Cell Res **221**(2): 261-71.

Fillmore, C. M., P. B. Gupta, et al. (2010). "Estrogen expands breast cancer stem-like cells through paracrine FGF/Tbx3 signaling." Proc Natl Acad Sci U S A.

Fischer, D. S., S. K. Chander, et al. (2003). "Novel D-ring modified steroid derivatives as potent, non-estrogenic, steroid sulfatase inhibitors with in vivo activity." J Steroid Biochem Mol Biol **84**(2-3): 343-9.

Fleming, J. S., C. R. Beaugie, et al. (2006). "Incessant ovulation, inflammation and epithelial ovarian carcinogenesis: revisiting old hypotheses." Mol Cell Endocrinol **247**(1-2): 4-21.

Flemming ID, C. J., Henson DE, Hutter RVP, Kennedy BJ & editors (1997). "Ovarian cancer staging" in American joint committee on cancer (AJCC) cancer staging manual. Published by Springer, USA. Page 275-85.

Flyvbjerg, A., O. Mogensen, et al. (1997). "Elevated serum insulin-like growth factor-binding protein 2 (IGFBP-2) and decreased IGFBP-3 in epithelial ovarian cancer: correlation with cancer antigen 125 and tumor-associated trypsin inhibitor." J Clin Endocrinol Metab **82**(7): 2308-13.

Foley, E. F., A. A. Jazaeri, et al. (2000). "Selective loss of estrogen receptor beta in malignant human colon." Cancer Res **60**(2): 245-8.

Foster, P. A., S. K. Chander, et al. (2008a). "A new therapeutic strategy against hormone-dependent breast cancer: the preclinical development of a dual aromatase and sulfatase inhibitor." Clin Cancer Res **14**(20): 6469-77.

Foster, P. A., S. K. Chander, et al. (2008b). "Efficacy of three potent steroid sulfatase inhibitors: pre-clinical investigations for their use in the treatment of hormone-dependent breast cancer." Breast Cancer Res Treat **111**(1): 129-38.

Franceschi, S., C. La Vecchia, et al. (1991). "Pooled analysis of 3 European case-control studies of ovarian cancer: II. Age at menarche and at menopause." Int J Cancer **49**(1): 57-60.

Fujimura, M., T. Hidaka, et al. (2001). "Absence of estrogen receptor-alpha expression in human ovarian clear cell adenocarcinoma compared with ovarian serous, endometrioid, and mucinous adenocarcinoma." Am J Surg Pathol **25**(5): 667-72.

Galtier-Dereure, F., F. Capony, et al. (1992). "Estradiol stimulates cell growth and secretion of procathepsin D and a 120-kilodalton protein in the human ovarian cancer cell line BG-1." J Clin Endocrinol Metab **75**(6): 1497-502.

Gerard, N., M. Caillaud, et al. (2004). "The interleukin-1 system and female reproduction." J Endocrinol **180**(2): 203-12.

Gilfillan, C. P., D. M. Robertson, et al. (1996). "The control of ovulation in mothers of dizygotic twins." J Clin Endocrinol Metab **81**(4): 1557-62.

Giuntoli, R. L., 2nd, T. J. Webb, et al. (2009). "Ovarian cancer-associated ascites demonstrates altered immune environment: implications for antitumor immunity." Anticancer Res **29**(8): 2875-84.

Godwin, A. K., J. R. Testa, et al. (1992). "Spontaneous transformation of rat ovarian surface epithelial cells: association with cytogenetic changes and implications of repeated ovulation in the etiology of ovarian cancer." J Natl Cancer Inst **84**(8): 592-601.

Grady, D., T. Gebretsadik, et al. (1995). "Hormone replacement therapy and endometrial cancer risk: a meta-analysis." Obstet Gynecol **85**(2): 304-13.

Grundker, C. and G. Emons (2003). "Role of gonadotropin-releasing hormone (GnRH) in ovarian cancer." Reprod Biol Endocrinol **1**: 65.

Gubbay, O., W. Guo, et al. (2004). "Anti-inflammatory and proliferative responses in human and ovine ovarian surface epithelial cells." Reproduction **128**(5): 607-14.

Gubbay, O., W. Guo, et al. (2005). "Inflammation-associated gene expression is altered between normal human ovarian surface epithelial cells and cell lines derived from ovarian adenocarcinomas." Br J Cancer **92**(10): 1927-33.

Gunnarsson, C., E. Hellqvist, et al. (2005). "17beta-Hydroxysteroid dehydrogenases involved in local oestrogen synthesis have prognostic significance in breast cancer." Br J Cancer **92**(3): 547-52.

Gustafsson, J. A. (1999). "Estrogen receptor beta--a new dimension in estrogen mechanism of action." J Endocrinol **163**(3): 379-83.

Hannum, C. H., C. J. Wilcox, et al. (1990). "Interleukin-1 receptor antagonist activity of a human interleukin-1 inhibitor." Nature **343**(6256): 336-40.

Hashii, K., H. Fujiwara, et al. (1998). "Peripheral blood mononuclear cells stimulate progesterone production by luteal cells derived from pregnant and non-pregnant women: possible involvement of interleukin-4 and interleukin-10 in corpus luteum function and differentiation." Hum Reprod **13**(1O): 2738-44.

Hayashido, Y., A. Lucas, et al. (1998). "Estradiol and fibulin-1 inhibit motility of human ovarian- and breast-cancer cells induced by fibronectin." Int J Cancer **75**(4): 654-8.

Helzlsouer, K. J., A. J. Alberg, et al. (1995). "Serum gonadotropins and steroid hormones and the development of ovarian cancer." JAMA **274**(24): 1926-30.

Hillier, S. G., R. A. Anderson, et al. (1998). "Expression of oestrogen receptor alpha and beta in cultured human ovarian surface epithelial cells." Mol Hum Reprod **4**(8): 811-5.

Ho, S. M., K. M. Lau, et al. (2003). "Profiling follicle stimulating hormone-induced gene expression changes in normal and malignant human ovarian surface epithelial cells." Oncogene **22**(27): 4243-56.

Ho, Y. T., A. Purohit, et al. (2003). "Inhibition of carbonic anhydrase II by steroidal and non-steroidal sulphamates." Biochem Biophys Res Commun **305**(4): 909-14.

Hodge, D. R., E. M. Hurt, et al. (2005). "The role of IL-6 and STAT3 in inflammation and cancer." Eur J Cancer **41**(16): 2502-12.

Holmes, W. E., J. Lee, et al. (1991). "Structure and functional expression of a human interleukin-8 receptor." Science **253**(5025): 1278-80.

Honma, S., K. Shimodaira, et al. (2002). "The influence of inflammatory cytokines on estrogen production and cell proliferation in human breast cancer cells." Endocr J **49**(3): 371-7.

Horii, Y., H. Takei, et al. (2006). "The regulatory effect of tamoxifen on fibronectin expression in estrogen-dependent MCF-7 breast carcinoma cells." Oncol Rep **15**(5): 1191-5.

Horvath, L. G., S. M. Henshall, et al. (2001). "Frequent loss of estrogen receptor-beta expression in prostate cancer." Cancer Res **61**(14): 5331-5.

Hua, W., T. Christianson, et al. (1995). "SKOV3 ovarian carcinoma cells have functional estrogen receptor but are growth-resistant to estrogen and antiestrogens." J Steroid Biochem Mol Biol **55**(3-4): 279-89.

Huleihel, M., E. Maymon, et al. (1997). "Distinct patterns of expression of interleukin-1 alpha and beta by normal and cancerous human ovarian tissues." Eur Cytokine Netw **8**(2): 179-87.

Humpel, M., U. Tuber, et al. (1990). "Comparison of serum ethinyl estradiol, sex-hormone-binding globulin, corticoid-binding globulin and cortisol levels in women using two low-dose combined oral contraceptives." Horm Res **33**(1): 35-9.

Huynh, H., X. Yang, et al. (1996). "Estradiol and antiestrogens regulate a growth inhibitory insulin-like growth factor binding protein 3 autocrine loop in human breast cancer cells." J Biol Chem **271**(2): 1016-21.

Imai, A., T. Ohno, et al. (1994). "Lack of evidence for aromatase expression in human ovarian epithelial carcinoma." Ann Clin Biochem **31 (Pt 1)**: 65-71.

Ivarsson, K., A. Ekerydh, et al. (2000). "Upregulation of interleukin-8 and polarized epithelial expression of interleukin-8 receptor A in ovarian carcinomas." Acta Obstet Gynecol Scand **79**(9): 777-84.

Ivarsson, K., E. Runesson, et al. (1998). "The chemotactic cytokine interleukin-8--a cyst fluid marker for malignant epithelial ovarian cancer?" Gynecol Oncol **71**(3): 420-3.

Ivarsson, K., K. Sundfeldt, et al. (2001). "Diverse effects of FSH and LH on proliferation of human ovarian surface epithelial cells." Hum Reprod **16**(1): 18-23.

Jasper, M. J., M. Brannstrom, et al. (1996). "Granulocyte-macrophage colony-stimulating factor: presence in human follicular fluid, protein secretion and mRNA expression by ovarian cells." Mol Hum Reprod **2**(8): 555-62.

Ji, Q., P. I. Liu, et al. (2004). "Follicle stimulating hormone-induced growth promotion and gene expression profiles on ovarian surface epithelial cells." Int J Cancer **112**(5): 803-14.

Johnson, M. R., A. Abbas, et al. (1994). "Maternal plasma levels of human chorionic gonadotrophin, oestradiol and progesterone in multifetal pregnancies before and after fetal reduction." J Endocrinol **143**(2): 309-12.

Johnston, S. R. (2004). "Ovarian cancer: review of the National Institute for Clinical Excellence (NICE) guidance recommendations." Cancer Invest **22**(5): 730-42.

Kabawat, S. E., R. C. Bast, Jr., et al. (1983). "Tissue distribution of a coelomic-epithelium-related antigen recognized by the monoclonal antibody OC125." Int J Gynecol Pathol **2**(3): 275-85.

Kaga, K., H. Sasano, et al. (1996). "Aromatase in human common epithelial ovarian neoplasms." Am J Pathol **149**(1): 45-51.

Kang, S. K., K. W. Cheng, et al. (2000). "Differential expression of human gonadotropin-releasing hormone receptor gene in pituitary and ovarian cells." Mol Cell Endocrinol **162**(1-2): 157-66.

Karlan, B. Y., J. Jones, et al. (1995). "Steroid hormone effects on the proliferation of human ovarian surface epithelium in vitro." Am J Obstet Gynecol **173**(1): 97-104.

Karst, A. M. and R. Drapkin (2010). "Ovarian cancer pathogenesis: a model in evolution." J Oncol **2010**: 932371.

Katsaros, D., H. Yu, et al. (2001). "IGFBP-3 in epithelial ovarian carcinoma and its association with clinico-pathological features and patient survival." Eur J Cancer **37**(4): 478-85.

Kawakami, Y., N. Nagai, et al. (1997). "Interleukin-1 as an autocrine stimulator in the growth of human ovarian cancer cells." Hiroshima J Med Sci **46**(1): 51-9.

Kaye, S. B. (1996). "Ovarian cancer, from the laboratory to the clinic: challenges for the future." Ann Oncol **7**(1): 9-13.

Khan, S. A., K. Schmidt, et al. (1988). "Human testis cytosol and ovarian follicular fluid contain high amounts of interleukin-1-like factor(s)." Mol Cell Endocrinol **58**(2-3): 221-30.

Killinger, D. W., E. Perel, et al. (1987). "The relationship between aromatase activity and body fat distribution." Steroids **50**(1-3): 61-72.

Kioi, M., S. Takahashi, et al. (2005). "Expression and targeting of interleukin-4 receptor for primary and advanced ovarian cancer therapy." Cancer Res **65**(18): 8388-96.

Kitawaki, J., T. Noguchi, et al. (1996). "Immunohistochemical localisation of aromatase and its correlation with progesterone receptors in ovarian epithelial tumours." Anticancer Res **16**(1): 91-7.

Kondera-Anasz, Z., A. Mielczarek-Palacz, et al. (2003). "Significantly increased interleukin-1A and interleukin-1 soluble type II receptor levels in women with ovarian cancer." Ginekol Pol **74**(9): 761-6.

Kotenko, S. V., C. D. Krause, et al. (1997). "Identification and functional characterization of a second chain of the interleukin-10 receptor complex." Embo J **16**(19): 5894-903.

Krywicki, R. F., J. A. Figueroa, et al. (1993). "Regulation of insulin-like growth factor binding proteins in ovarian cancer cells by oestrogen." Eur J Cancer **29A**(14): 2015-9.

Ksiazek, K., J. Mikula-Pietrasik, et al. (2009). "Senescent peritoneal mesothelial cells promote ovarian cancer cell adhesion: the role of oxidative stress-induced fibronectin." Am J Pathol **174**(4): 1230-40.

Kubba, A. (1995). "New HRT options in general practice." Practitioner **239**(1552): 435-9.

Kumle, M., E. Weiderpass, et al. (2004). "Risk for invasive and borderline epithelial ovarian neoplasias following use of hormonal contraceptives: the Norwegian-Swedish Women's Lifestyle and Health Cohort Study." Br J Cancer **90**(7): 1386-91.

Kuroda, H., M. Mandai, et al. (2001). "Human ovarian surface epithelial (OSE) cells express LH/hCG receptors, and hCG inhibits apoptosis of OSE cells via up-regulation of insulin-like growth factor-1." Int J Cancer **91**(3): 309-15.

Lacey, J. V., Jr., L. A. Brinton, et al. (2006). "Menopausal hormone therapy and ovarian cancer risk in the National Institutes of Health-AARP Diet and Health Study Cohort." J Natl Cancer Inst **98**(19): 1397-405.

Lacey, J. V., Jr., P. J. Mink, et al. (2002). "Menopausal hormone replacement therapy and risk of ovarian cancer." Jama **288**(3): 334-41.

Lambalk, C. B., D. I. Boomsma, et al. (1998). "Increased levels and pulsatility of follicle-stimulating hormone in mothers of hereditary dizygotic twins." J Clin Endocrinol Metab **83**(2): 481-6.

Langdon, S. P., M. M. Hawkes, et al. (1990). "Oestrogen receptor expression and the effects of oestrogen and tamoxifen on the growth of human ovarian carcinoma cell lines." Br J Cancer **62**(2): 213-6.

Lau, K. M., S. C. Mok, et al. (1999). "Expression of human estrogen receptor-alpha and -beta, progesterone receptor, and androgen receptor mRNA in normal and malignant ovarian epithelial cells." Proc Natl Acad Sci U S A **96**(10): 5722-7.

Laviolette, L. A., K. Garson, et al. (2010). "17beta-estradiol accelerates tumor onset and decreases survival in a transgenic mouse model of ovarian cancer." Endocrinology **151**(3): 929-38.

Lee, H. Y., K. H. Chun, et al. (2002). "Insulin-like growth factor binding protein-3 inhibits the growth of non-small cell lung cancer." Cancer Res **62**(12): 3530-7.

Lee, P., D. G. Rosen, et al. (2005). "Expression of progesterone receptor is a favorable prognostic marker in ovarian cancer." Gynecol Oncol **96**(3): 671-7.

Li, A. J., R. L. Baldwin, et al. (2003). "Estrogen and progesterone receptor subtype expression in normal and malignant ovarian epithelial cell cultures." Am J Obstet Gynecol **189**(1): 22-7.

Li, D., J. Ding, et al. (2009). "Fibronectin promotes tyrosine phosphorylation of paxillin and cell invasiveness in the gastric cancer cell line AGS." Tumori **95**(6): 769-79.

Lin, H. K., S. Steckelbroeck, et al. (2004). "Characterization of a monoclonal antibody for human aldo-keto reductase AKR1C3 (type 2 3alpha-hydroxysteroid dehydrogenase/type 5 17beta-hydroxysteroid dehydrogenase); immunohistochemical detection in breast and prostate." Steroids **69**(13-14): 795-801.

Lindgren, P., T. Backstrom, et al. (2001). "Steroid receptors and hormones in relation to cell proliferation and apoptosis in poorly differentiated epithelial ovarian tumors." Int J Oncol **19**(1): 31-8.

Lindgren, P. R., S. Cajander, et al. (2004). "Estrogen and progesterone receptors in ovarian epithelial tumors." Mol Cell Endocrinol **221**(1-2): 97-104.

Liu, J., R. W. Rebar, et al. (1983). "Neuroendocrine control of the postpartum period." Clin Perinatol **10**(3): 723-36.

Lobo, R. A., G. S. diZerega, et al. (1985). "Follicular fluid steroid levels in dysmature and mature follicles from spontaneous and hyperstimulated cycles in normal and anovulatory women." J Clin Endocrinol Metab **60**(1): 81-7.

Lu, L., D. Katsaros, et al. (2006). "The relationship of insulin-like growth factor-II, insulin-like growth factor binding protein-3, and estrogen receptor-alpha expression to disease progression in epithelial ovarian cancer." Clin Cancer Res **12**(4): 1208-14.

Ma, J., M. N. Pollak, et al. (1999). "Prospective study of colorectal cancer risk in men and plasma levels of insulin-like growth factor (IGF)-I and IGF-binding protein-3." J Natl Cancer Inst **91**(7): 620-5.

Machelon, V., F. Nome, et al. (1994). "Comparative IL-6 effects on FSH and hCG-induced functions in porcine granulosa cell cultures." Cell Mol Biol (Noisy-le-grand) **40**(3): 373-80.

Marth, C., A. G. Zeimet, et al. (1996). "Different effects of interferons, interleukin-1beta and tumor necrosis factor-alpha in normal (OSE) and malignant human ovarian epithelial cells." Int J Cancer **67**(6): 826-30.

Martin, N. G., D. M. Robertson, et al. (1991). "Elevation of follicular phase inhibin and luteinizing hormone levels in mothers of dizygotic twins suggests nonovarian control of human multiple ovulation." Fertil Steril **56**(3): 469-74.

Matsuoka, R., A. Yanaihara, et al. (2002). "Regulation of estrogen activity in human endometrium: effect of IL-1beta on steroid sulfatase activity in human endometrial stromal cells." Steroids **67**(7): 655-9.

McDonnel, A. C. and W. J. Murdoch (2001). "High-dose progesterone inhibition of urokinase secretion and invasive activity by SKOV-3 ovarian carcinoma cells: evidence for a receptor-independent nongenomic effect on the plasma membrane." J Steroid Biochem Mol Biol **78**(2): 185-91.

McDonnel, A. C., E. A. Van Kirk, et al. (2005). "Effects of progesterone on ovarian tumorigenesis in xenografted mice." Cancer Lett **221**(1): 49-53.

McKenna, N. J., R. B. Lanz, et al. (1999). "Nuclear receptor coregulators: cellular and molecular biology." Endocr Rev **20**(3): 321-44.

McMahan, C. J., J. L. Slack, et al. (1991). "A novel IL-1 receptor, cloned from B cells by mammalian expression, is expressed in many cell types." Embo J **10**(10): 2821-32.

Merogi, A. J., A. J. Marrogi, et al. (1997). "Tumor-host interaction: analysis of cytokines, growth factors, and tumor-infiltrating lymphocytes in ovarian carcinomas." Hum Pathol **28**(3): 321-31.

Merritt, W. M., Y. G. Lin, et al. (2008). "Effect of interleukin-8 gene silencing with liposome-encapsulated small interfering RNA on ovarian cancer cell growth." J Natl Cancer Inst **100**(5): 359-72.

Miller, W. R. (2004). "Biological rationale for endocrine therapy in breast cancer." Best Pract Res Clin Endocrinol Metab **18**(1): 1-32.

Mittal, K. R., A. Zeleniuch-Jacquotte, et al. (1993). "Contralateral ovary in unilateral ovarian carcinoma: a search for preneoplastic lesions." Int J Gynecol Pathol **12**(1): 59-63.

Modan, B., P. Hartge, et al. (2001). "Parity, oral contraceptives, and the risk of ovarian cancer among carriers and noncarriers of a BRCA1 or BRCA2 mutation." N Engl J Med **345**(4): 235-40.

Moradi, M. M., L. F. Carson, et al. (1993). "Serum and ascitic fluid levels of interleukin-1, interleukin-6, and tumor necrosis factor-alpha in patients with ovarian epithelial cancer." Cancer **72**(8): 2433-40.

Murdoch, W. J., D. C. Colgin, et al. (1997). "Role of tumor necrosis factor-alpha in the ovulatory mechanism of ewes." J Anim Sci **75**(6): 1601-5.

Murdoch, W. J. and S. A. Lund (1999). "Prostaglandin-independent anovulatory mechanism of indomethacin action: inhibition of tumor necrosis factor alpha-induced sheep ovarian cell apoptosis." Biol Reprod **61**(6): 1655-9.

Murdoch, W. J. and A. C. McDonnel (2002). "Roles of the ovarian surface epithelium in ovulation and carcinogenesis." Reproduction **123**(6): 743-50.

Nakamura, Y., T. Suzuki, et al. (2006). "Steroid sulfatase and estrogen sulfotransferase in human prostate cancer." Prostate **66**(9): 1005-12.

Nash, M. A., R. Lenzi, et al. (1998). "Differential expression of cytokine transcripts in human epithelial ovarian carcinoma by solid tumour specimens, peritoneal exudate cells containing tumour, tumour-infiltrating lymphocyte (TIL)-derived T cell lines and established tumour cell lines." Clin Exp Immunol **112**(2): 172-80.

Negri, E., A. Tzonou, et al. (1999). "Hormonal therapy for menopause and ovarian cancer in a collaborative re-analysis of European studies." Int J Cancer **80**(6): 848-51.

Nelms, K., A. D. Keegan, et al. (1999). "The IL-4 receptor: signaling mechanisms and biologic functions." Annu Rev Immunol **17**: 701-38.

Ness, R. B. and C. Cottreau (1999). "Possible role of ovarian epithelial inflammation in ovarian cancer." J Natl Cancer Inst **91**(17): 1459-67.

Ness, R. B., J. A. Grisso, et al. (2000). "Factors related to inflammation of the ovarian epithelium and risk of ovarian cancer." Epidemiology **11**(2): 111-7.

Nicosia, S. V. and J. H. Johnson (1984). "Surface morphology of ovarian mesothelium (surface epithelium) and of other pelvic and extrapelvic mesothelial sites in the rabbit." Int J Gynecol Pathol **3**(3): 249-60.

Noguchi, T., J. Kitawaki, et al. (1993). "Relationship between aromatase activity and steroid receptor levels in ovarian tumors from postmenopausal women." J Steroid Biochem Mol Biol **44**(4-6): 657-60.

Nowak, M., E. Glowacka, et al. "Proinflammatory and immunosuppressive serum, ascites and cyst fluid cytokines in patients with early and advanced ovarian cancer and benign ovarian tumors." Neuro Endocrinol Lett **31**(3): 375-83.

Nozawa, T., M. Suzuki, et al. (2004). "Involvement of estrone-3-sulfate transporters in proliferation of hormone-dependent breast cancer cells." J Pharmacol Exp Ther **311**(3): 1032-7.

Nozawa, T., M. Suzuki, et al. (2005). "Suppression of cell proliferation by inhibition of estrone-3-sulfate transporter in estrogen-dependent breast cancer cells." Pharm Res **22**(10): 1634-41.

O'Donnell, A. J., K. G. Macleod, et al. (2005). "Estrogen receptor-alpha mediates gene expression changes and growth response in ovarian cancer cells exposed to estrogen." Endocr Relat Cancer **12**(4): 851-66.

Obata, N. H., K. Tamakoshi, et al. (1997). "Effects of interleukin-6 on in vitro cell attachment, migration and invasion of human ovarian carcinoma." Anticancer Res **17**(1A): 337-42.

Oduwole, O. O., Y. Li, et al. (2004). "17beta-hydroxysteroid dehydrogenase type 1 is an independent prognostic marker in breast cancer." Cancer Res **64**(20): 7604-9.

Oesterreich, S., W. Deng, et al. (2003). "Estrogen-mediated down-regulation of E-cadherin in breast cancer cells." Cancer Res **63**(17): 5203-8.

Offner, F. A., P. Obrist, et al. (1995). "IL-6 secretion by human peritoneal mesothelial and ovarian cancer cells." Cytokine **7**(6): 542-7.

Oh, Y., H. L. Muller, et al. (1993). "Insulin-like growth factor binding protein (IGFBP)-3 levels in conditioned media of Hs578T human breast cancer cells are post-transcriptionally regulated." Growth Regul **3**(1): 84-7.

Okamura, H. and H. Katabuchi (2001). "Detailed morphology of human ovarian surface epithelium focusing on its metaplastic and neoplastic capability." Ital J Anat Embryol **106**(2 Suppl 2): 263-76.

Okamura, H., H. Katabuchi, et al. (2006). "Structural changes and cell properties of human ovarian surface epithelium in ovarian pathophysiology." Microsc Res Tech **69**(6): 469-81.

Okubo, T., S. C. Mok, et al. (2000). "Regulation of aromatase expression in human ovarian surface epithelial cells." J Clin Endocrinol Metab **85**(12): 4889-99.

Okuda, T., H. Saito, et al. (2001). "Steroid sulfatase expression in ovarian clear cell adenocarcinoma: immunohistochemical study." Gynecol Oncol **82**(3): 427-34.

Ozasa, H., T. Tominaga, et al. (1981). "Lysyl oxidase activity in the mouse uterine cervix is physiologically regulated by estrogen." Endocrinology **109**(2): 618-21.

Pankov, R. and K. M. Yamada (2002). "Fibronectin at a glance." J Cell Sci **115**(Pt 20): 3861-3.

Papacleovoulou, G., R. J. Edmondson, et al. (2009). "3beta-Hydroxysteroid dehydrogenases and pre-receptor steroid metabolism in the human ovarian surface epithelium." Mol Cell Endocrinol **301**(1-2): 65-73.

Papadimitriou, C. A., S. Markaki, et al. (2004). "Hormonal therapy with letrozole for relapsed epithelial ovarian cancer. Long-term results of a phase II study." Oncology **66**(2): 112-7.

Park, S. H., L. W. Cheung, et al. (2008). "Estrogen regulates Snail and Slug in the down-regulation of E-cadherin and induces metastatic potential of ovarian cancer cells through estrogen receptor alpha." Mol Endocrinol **22**(9): 2085-98.

Penning, T. M., M. E. Burczynski, et al. (2000). "Human 3alpha-hydroxysteroid dehydrogenase isoforms (AKR1C1-AKR1C4) of the aldo-keto reductase superfamily: functional plasticity and tissue distribution reveals roles in the inactivation and formation of male and female sex hormones." Biochem J **351**(Pt 1): 67-77.

Perez-Gracia, J. L. and E. M. Carrasco (2002). "Tamoxifen therapy for ovarian cancer in the adjuvant and advanced settings: systematic review of the literature and implications for future research." Gynecol Oncol **84**(2): 201-9.

Perez Gutthann, S., L. A. Garcia Rodriguez, et al. (1997). "Hormone replacement therapy and risk of venous thromboembolism: population based case-control study." BMJ **314**(7083): 796-800.

Peyrol, S., M. Raccurt, et al. (1997). "Lysyl oxidase gene expression in the stromal reaction to in situ and invasive ductal breast carcinoma." Am J Pathol **150**(2): 497-507.

Pinto, P. I., P. B. Singh, et al. (2006). "ICI 182,780 has agonistic effects and synergizes with estradiol-17 beta in fish liver, but not in testis." Reprod Biol Endocrinol **4**: 67.

Pisa, P., E. Halapi, et al. (1992). "Selective expression of interleukin 10, interferon gamma, and granulocyte-macrophage colony-stimulating factor in ovarian cancer biopsies." Proc Natl Acad Sci U S A **89**(16): 7708-12.

Pizzagalli, F., Z. Varga, et al. (2003). "Identification of steroid sulfate transport processes in the human mammary gland." J Clin Endocrinol Metab **88**(8): 3902-12.

Pratt, S. E. and M. N. Pollak (1993). "Estrogen and antiestrogen modulation of MCF7 human breast cancer cell proliferation is associated with specific alterations in accumulation of insulin-like growth factor-binding proteins in conditioned media." Cancer Res **53**(21): 5193-8.

Provost, P. R., P. H. Lima, et al. (2010). "A useful cell system for studying the regulation of 17HSD/KSR type 2 activity and expression in ovarian epithelial cancer." J Steroid Biochem Mol Biol.

Pujol, P., J. M. Rey, et al. (1998). "Differential expression of estrogen receptor-alpha and -beta messenger RNAs as a potential marker of ovarian carcinogenesis." Cancer Res **58**(23): 5367-73.

Purdie, D., A. Green, et al. (1995). "Reproductive and other factors and risk of epithelial ovarian cancer: an Australian case-control study. Survey of Women's Health Study Group." Int J Cancer **62**(6): 678-84.

Purdie, D. M., C. J. Bain, et al. (2003). "Ovulation and risk of epithelial ovarian cancer." Int J Cancer **104**(2): 228-32.

Purohit, A., L. W. Woo, et al. (2000). "In vivo inhibition of estrone sulfatase activity and growth of nitrosomethylurea-induced mammary tumors by 667 COUMATE." Cancer Res **60**(13): 3394-6.

Quinn, M. A., P. Pearce, et al. (1982). "Cytoplasmic steroid receptors in ovarian tumours." Br J Obstet Gynaecol **89**(9): 754-9.

Rae, M. T. and S. G. Hillier (2005). "Steroid signalling in the ovarian surface epithelium." Trends Endocrinol Metab **16**(7): 327-33.

Rae, M. T., D. Niven, et al. (2004a). "Antiinflammatory steroid action in human ovarian surface epithelial cells." J Clin Endocrinol Metab **89**(9): 4538-44.

Rae, M. T., D. Niven, et al. (2004b). "Steroid signalling in human ovarian surface epithelial cells: the response to interleukin-1alpha determined by microarray analysis." J Endocrinol **183**(1): 19-28.

Ranadive, G. N., J. S. Mistry, et al. (1998). "Rapid, convenient radioimmunoassay of estrone sulfate." Clin Chem **44**(2): 244-9.

Rao, B. R. and B. J. Slotman (1991). "Endocrine factors in common epithelial ovarian cancer." Endocr Rev **12**(1): 14-26.

Reed, M. J. and A. Purohit (1997). "Breast cancer and the role of cytokines in regulating estrogen synthesis: an emerging hypothesis." Endocr Rev **18**(5): 701-15.

Reed, M. J., A. Purohit, et al. (2005). "Steroid sulfatase: molecular biology, regulation, and inhibition." Endocr Rev **26**(2): 171-202.

Ren, C., G. Yang, et al. (1998). "Reduced lysyl oxidase messenger RNA levels in experimental and human prostate cancer." Cancer Res **58**(6): 1285-90.

Renehan, A. G., J. E. Painter, et al. (2001). "High-risk colorectal adenomas and serum insulin-like growth factors." Br J Surg **88**(1): 107-13.

Ricort, J. M. and M. Binoux (2002). "Insulin-like growth factor-binding protein-3 activates a phosphotyrosine phosphatase. Effects on the insulin-like growth factor signaling pathway." J Biol Chem **277**(22): 19448-54.

Riman, T., P. W. Dickman, et al. (2002). "Hormone replacement therapy and the risk of invasive epithelial ovarian cancer in Swedish women." J Natl Cancer Inst **94**(7): 497-504.

Riman, T., S. Nilsson, et al. (2004). "Review of epidemiological evidence for reproductive and hormonal factors in relation to the risk of epithelial ovarian malignancies." Acta Obstet Gynecol Scand **83**(9): 783-95.

Risch, H. A. (1998). "Hormonal etiology of epithelial ovarian cancer, with a hypothesis concerning the role of androgens and progesterone." J Natl Cancer Inst **90**(23): 1774-86.

Risch, H. A., L. D. Marrett, et al. (1994). "Parity, contraception, infertility, and the risk of epithelial ovarian cancer." Am J Epidemiol **140**(7): 585-97.

Risch, H. A., J. R. McLaughlin, et al. (2006). "Population BRCA1 and BRCA2 mutation frequencies and cancer penetrances: a kin-cohort study in Ontario, Canada." J Natl Cancer Inst **98**(23): 1694-706.

Robertson, J. A., Y. Zhang, et al. (2001). "ICI 182,780 acts as a partial agonist and antagonist of estradiol effects in specific cells of the sheep uterus." J Steroid Biochem Mol Biol **77**(4-5): 281-7.

Roby, K. F., C. C. Taylor, et al. (2000). "Development of a syngeneic mouse model for events related to ovarian cancer." Carcinogenesis **21**(4): 585-91.

Rochefort, H., N. Platet, et al. (1998). "Estrogen receptor mediated inhibition of cancer cell invasion and motility: an overview." J Steroid Biochem Mol Biol **65**(1-6): 163-8.

Rodriguez, C., A. V. Patel, et al. (2001). "Estrogen replacement therapy and ovarian cancer mortality in a large prospective study of US women." Jama **285**(11): 1460-5.

Roger, P., M. E. Sahla, et al. (2001). "Decreased expression of estrogen receptor beta protein in proliferative preinvasive mammary tumors." Cancer Res **61**(6): 2537-41.

Rossing, M. A., J. R. Daling, et al. (1994). "Ovarian tumors in a cohort of infertile women." N Engl J Med **331**(12): 771-6.

Runesson, E., E. K. Bostrom, et al. (1996). "The human preovulatory follicle is a source of the chemotactic cytokine interleukin-8." Mol Hum Reprod **2**(4): 245-50.

Runesson, E., K. Ivarsson, et al. (2000). "Gonadotropin- and cytokine-regulated expression of the chemokine interleukin 8 in the human preovulatory follicle of the menstrual cycle." J Clin Endocrinol Metab **85**(11): 4387-95.

Salazar-Martinez, E., E. C. Lazcano-Ponce, et al. (1999). "Reproductive factors of ovarian and endometrial cancer risk in a high fertility population in Mexico." Cancer Res **59**(15): 3658-62.

Samani, A. A., S. Yakar, et al. (2007). "The role of the IGF system in cancer growth and metastasis: overview and recent insights." Endocr Rev **28**(1): 20-47.

Sanada, H., J. Shikata, et al. (1978). "Changes in collagen cross-linking and lysyl oxidase by estrogen." Biochim Biophys Acta **541**(3): 408-13.

Sasano, H., T. J. Anderson, et al. (2005). "The validation of new aromatase monoclonal antibodies for immunohistochemistry--a correlation with biochemical activities in 46 cases of breast cancer." J Steroid Biochem Mol Biol **95**(1-5): 35-9.

Sasano, H., A. R. Frost, et al. (1996). "Aromatase and 17 beta-hydroxysteroid dehydrogenase type 1 in human breast carcinoma." J Clin Endocrinol Metab **81**(11): 4042-6.

Sato, R., T. Suzuki, et al. (2009). "Steroid sulfatase and estrogen sulfotransferase in colon carcinoma: regulators of intratumoral estrogen concentrations and potent prognostic factors." Cancer Res **69**(3): 914-22.

Schiffenbauer, Y. S., R. Abramovitch, et al. (1997). "Loss of ovarian function promotes angiogenesis in human ovarian carcinoma." Proc Natl Acad Sci U S A **94**(24): 13203-8.

Schiffenbauer, Y. S., G. Meir, et al. (2002). "Gonadotropin stimulation of MLS human epithelial ovarian carcinoma cells augments cell adhesion mediated by CD44 and by alpha(v)-integrin." Gynecol Oncol **84**(2): 296-302.

Schildkraut, J. M., B. Calingaert, et al. (2002). "Impact of progestin and estrogen potency in oral contraceptives on ovarian cancer risk." J Natl Cancer Inst **94**(1): 32-8.

Schildkraut, J. M., P. J. Schwingl, et al. (1996). "Epithelial ovarian cancer risk among women with polycystic ovary syndrome." Obstet Gynecol **88**(4 Pt 1): 554-9.

Semb, H. and G. Christofori (1998). "The tumor-suppressor function of E-cadherin." Am J Hum Genet **63**(6): 1588-93.

Shoham, Z. (1994). "Epidemiology, etiology, and fertility drugs in ovarian epithelial carcinoma: where are we today?" Fertil Steril **62**(3): 433-48.

Shu, X. O., L. A. Brinton, et al. (1989). "Population-based case-control study of ovarian cancer in Shanghai." Cancer Res **49**(13): 3670-4.

Silva, E. G., C. Tornos, et al. (1998). "Induction of epithelial neoplasms in the ovaries of guinea pigs by estrogenic stimulation." Gynecol Oncol **71**(2): 240-6.

Silva, E. G., C. Tornos, et al. (1997). "The induction of benign epithelial neoplasms of the ovaries of guinea pigs by testosterone stimulation: a potential animal model." Mod Pathol **10**(9): 879-83.

Smith-Mungo, L. I. and H. M. Kagan (1998). "Lysyl oxidase: properties, regulation and multiple functions in biology." Matrix Biol **16**(7): 387-98.

Smyth, J. F., C. Gourley, et al. (2007). "Antiestrogen therapy is active in selected ovarian cancer cases: the use of letrozole in estrogen receptor-positive patients." Clin Cancer Res **13**(12): 3617-22.

Sommer, S. and S. A. Fuqua (2001). "Estrogen receptor and breast cancer." Semin Cancer Biol **11**(5): 339-52.

Song, J., A. Fadiel, et al. (2005). "Estradiol-induced ezrin overexpression in ovarian cancer: a new signaling domain for estrogen." Cancer Lett **220**(1): 57-65.

Song, W. C., Y. Qian, et al. (1998). "Estrogen sulfotransferase expression in the human liver: marked interindividual variation and lack of gender specificity." J Pharmacol Exp Ther **284**(3): 1197-202.

Song, W. C., Y. Qian, et al. (1997). "Cellular localization and regulation of expression of testicular estrogen sulfotransferase." Endocrinology **138**(11): 5006-12.

Spits, H. and R. de Waal Malefyt (1992). "Functional characterization of human IL-10." Int Arch Allergy Immunol **99**(1): 8-15.

Stabile, L. P., A. L. Davis, et al. (2002). "Human non-small cell lung tumors and cells derived from normal lung express both estrogen receptor alpha and beta and show biological responses to estrogen." Cancer Res **62**(7): 2141-50.

Stanway, S. J., A. Purohit, et al. (2006). "Phase I study of STX 64 (667 Coumate) in breast cancer patients: the first study of a steroid sulfatase inhibitor." Clin Cancer Res **12**(5): 1585-92.

Stratton, J. F., J. A. Tidy, et al. (2001). "The surgical management of ovarian cancer." Cancer Treat Rev **27**(2): 111-8.

Studd John and S. Roger (1998). "The menopause and hormone replacement therapy." 2nd edition, published by Martin Dunitz Ltd, London. Chapter 4.

Sturdee, D. W. (1997). "Newer HRT regimens." Br J Obstet Gynaecol **104**(10): 1109-15.

Suzuki, T., Y. Miki, et al. (2005). "Sex steroid-producing enzymes in human breast cancer." Endocr Relat Cancer **12**(4): 701-20.

Suzuki, T., T. Nakata, et al. (2003). "Estrogen sulfotransferase and steroid sulfatase in human breast carcinoma." Cancer Res **63**(11): 2762-70.

Syed, V., K. Mukherjee, et al. (2005). "Identification of ATF-3, caveolin-1, DLC-1, and NM23-H2 as putative antitumorigenic, progesterone-regulated genes for ovarian cancer cells by gene profiling." Oncogene **24**(10): 1774-87.

Syed, V., G. Ulinski, et al. (2002). "Reproductive hormone-induced, STAT3-mediated interleukin 6 action in normal and malignant human ovarian surface epithelial cells." J Natl Cancer Inst **94**(8): 617-29.

Syed, V., G. Ulinski, et al. (2001). "Expression of gonadotropin receptor and growth responses to key reproductive hormones in normal and malignant human ovarian surface epithelial cells." Cancer Res **61**(18): 6768-76.

Takeichi, M. (1995). "Morphogenetic roles of classic cadherins." Curr Opin Cell Biol **7**(5): 619-27.

Tashiro H, F. M., Miyazaki K, Okamura H. (1993). "Biological relevance and analysis of c-myc gene expression in normal human ovary." In: Leung PCK, Hsueh AJW, Friesen HG, editors. Molecular basis of reproductive endocrinology. New York: Springer-Verlag: 243–249.

Testa, J. R., L. A. Getts, et al. (1994). "Spontaneous transformation of rat ovarian surface epithelial cells results in well to poorly differentiated tumors with a parallel range of cytogenetic complexity." Cancer Res **54**(10): 2778-84.

Thomas, H. V., M. F. Murphy, et al. (1998). "Pregnancy and menstrual hormone levels in mothers of twins compared to mothers of singletons." Ann Hum Biol **25**(1): 69-75.

Thompson, M. A., M. D. Adelson, et al. (1988). "Aromatization of testosterone by epithelial tumor cells cultured from patients with ovarian carcinoma." Cancer Res **48**(22): 6491-7.

Titus-Ernstoff, L., K. Perez, et al. (2001). "Menstrual and reproductive factors in relation to ovarian cancer risk." Br J Cancer **84**(5): 714-21.

Tokunaga, H., J. Akahira, et al. (2007). "Ovarian epithelial carcinoma with estrogen-producing stroma." Pathol Int **57**(5): 285-90.

Tora, L., J. White, et al. (1989). "The human estrogen receptor has two independent nonacidic transcriptional activation functions." Cell **59**(3): 477-87.

Torbenson, M., J. Wang, et al. (2002). "Hepatocellular carcinomas show abnormal expression of fibronectin protein." Mod Pathol **15**(8): 826-30.

Toutirais, O., P. Chartier, et al. (2003). "Constitutive expression of TGF-beta1, interleukin-6 and interleukin-8 by tumor cells as a major component of immune escape in human ovarian carcinoma." Eur Cytokine Netw **14**(4): 246-55.

Tumolo, S., B. R. Rao, et al. (1994). "Phase II trial of flutamide in advanced ovarian cancer: an EORTC Gynaecological Cancer Cooperative Group study." Eur J Cancer **30A**(7): 911-4.

Ugele, B., A. Bahn, et al. (2008). "Functional differences in steroid sulfate uptake of organic anion transporter 4 (OAT4) and organic anion transporting polypeptide 2B1 (OATP2B1) in human placenta." J Steroid Biochem Mol Biol **111**(1-2): 1-6.

van der Zee, A. G., E. M. de Cuyper, et al. (1995). "Higher levels of interleukin-6 in cystic fluids from patients with malignant versus benign ovarian tumors correlate with decreased hemoglobin levels and increased platelet counts." Cancer **75**(4): 1004-9.

van Niekerk, C. C., O. C. Boerman, et al. (1991). "Marker profile of different phases in the transition of normal human ovarian epithelium to ovarian carcinomas." Am J Pathol **138**(2): 455-63.

Vassilomanolakis, M., G. Koumakis, et al. (1997). "A phase II study of flutamide in ovarian cancer." Oncology **54**(3): 199-202.

Veatch, A. L., L. F. Carson, et al. (1994). "Differential expression of the cell-cell adhesion molecule E-cadherin in ascites and solid human ovarian tumor cells." Int J Cancer **58**(3): 393-9.

Veenhof, C. H., M. E. van der Burg, et al. (1994). "Phase II study of high-dose megestrol acetate in patients with advanced ovarian carcinoma." Eur J Cancer **30A**(5): 697-8.

Verschraegen, C. F., S. Westphalen, et al. (2003). "Phase II study of cetrorelix, a luteinizing hormone-releasing hormone antagonist in patients with platinum-resistant ovarian cancer." Gynecol Oncol **90**(3): 552-9.

Vierikko, P., A. Kauppila, et al. (1983). "Cytosol and nuclear estrogen and progestin receptors and 17 beta-hydroxysteroid dehydrogenase activity in non-diseased tissue and in benign and malignant tumors of the human ovary." Int J Cancer **32**(4): 413-22.

Vleminckx, K., L. Vakaet, Jr., et al. (1991). "Genetic manipulation of E-cadherin expression by epithelial tumor cells reveals an invasion suppressor role." Cell **66**(1): 107-19.

Vollmer, G., A. C. Hopert, et al. (1995). "Fibronectin is an estrogen-repressed protein in RUCA-I rat endometrial adenocarcinoma cells." J Steroid Biochem Mol Biol **54**(3-4): 131-9.

Waalkes, S., F. Atschekzei, et al. (2010). "Fibronectin 1 mRNA expression correlates with advanced disease in renal cancer." BMC Cancer **10**: 503.

Wang, L. J., M. Brannstrom, et al. (1992). "Tumor necrosis factor alpha in the human ovary: presence in follicular fluid and effects on cell proliferation and prostaglandin production." Fertil Steril **58**(5): 934-40.

Wang, L. J. and R. J. Norman (1992). "Concentrations of immunoreactive interleukin-1 and interleukin-2 in human preovulatory follicular fluid." Hum Reprod **7**(2): 147-50.

Watson, J. M., J. L. Sensintaffar, et al. (1990). "Constitutive production of interleukin 6 by ovarian cancer cell lines and by primary ovarian tumor cultures." Cancer Res **50**(21): 6959-65.

Webb, A. C., K. L. Collins, et al. (1986). "Interleukin-1 gene (IL1) assigned to long arm of human chromosome 2." Lymphokine Res **5**(2): 77-85.

Werness, B. A., A. M. Afify, et al. (1999). "Altered surface and cyst epithelium of ovaries removed prophylactically from women with a family history of ovarian cancer." Hum Pathol **30**(2): 151-7.

Whittaker, P. G., M. R. Morgan, et al. (1980). "Serum equilin, oestrone, and oestradiol levels in postmenopausal women receiving conjugated equine oestrogens ('Premarin')." Lancet **1**(8158): 14-6.

Whittemore, A. S., R. Harris, et al. (1992). "Characteristics relating to ovarian cancer risk: collaborative analysis of 12 US case-control studies. IV. The pathogenesis of epithelial ovarian cancer. Collaborative Ovarian Cancer Group." Am J Epidemiol **136**(10): 1212-20.

Wiley, A., D. Katsaros, et al. (2006). "Methylation of the insulin-like growth factor binding protein-3 gene and prognosis of epithelial ovarian cancer." Int J Gynecol Cancer **16**(1): 210-8.

Willcocks, D., M. Toppila, et al. (1983). "Estrogen and progesterone receptors in human ovarian tumors." Gynecol Oncol **16**(2): 246-53.

Williams, A. C., T. J. Collard, et al. (2000). "Increased p53-dependent apoptosis by the insulin-like growth factor binding protein IGFBP-3 in human colonic adenoma-derived cells." Cancer Res **60**(1): 22-7.

Williams, T. I., K. L. Toups, et al. (2007). "Epithelial ovarian cancer: disease etiology, treatment, detection, and investigational gene, metabolite, and protein biomarkers." J Proteome Res **6**(8): 2936-62.

Wimalasena, J., D. Meehan, et al. (1993). "Growth factors interact with estradiol and gonadotropins in the regulation of ovarian cancer cell growth and growth factor receptors." Oncol Res **5**(8): 325-37.

Wlcek, K., M. Svoboda, et al. (2008). "Altered expression of organic anion transporter polypeptide (OATP) genes in human breast carcinoma." Cancer Biol Ther **7**(9): 1450-5.

Wong, A. S. and P. C. Leung (2007). "Role of endocrine and growth factors on the ovarian surface epithelium." J Obstet Gynaecol Res **33**(1): 3-16.

Wooster, R. and B. L. Weber (2003). "Breast and ovarian cancer." N Engl J Med **348**(23): 2339-47.

Woznick, A. R., A. L. Braddock, et al. (2005). "Lysyl oxidase expression in bronchogenic carcinoma." Am J Surg **189**(3): 297-301.

Xie, K. (2001). "Interleukin-8 and human cancer biology." Cytokine Growth Factor Rev **12**(4): 375-91.

Xing, H., Y. Cao, et al. (2008). "Fibronectin-mediated activation of Akt2 protects human ovarian and breast cancer cells from docetaxel-induced apoptosis via inhibition of the p38 pathway." Apoptosis **13**(2): 213-23.

Yang, L., L. Wang, et al. (2003). "Interleukin-6 differentially regulates androgen receptor transactivation via PI3K-Akt, STAT3, and MAPK, three distinct signal pathways in prostate cancer cells." Biochem Biophys Res Commun **305**(3): 462-9.

Yang, W. L., A. K. Godwin, et al. (2004). "Tumor necrosis factor-alpha-induced matrix proteolytic enzyme production and basement membrane remodeling

by human ovarian surface epithelial cells: molecular basis linking ovulation and cancer risk." Cancer Res **64**(4): 1534-40.

Yang, Z., X. Zhang, et al. (2007). "Up-regulation of gastric cancer cell invasion by Twist is accompanied by N-cadherin and fibronectin expression." Biochem Biophys Res Commun **358**(3): 925-30.

Yong, P. Y., C. Harlow, et al. (2002). "Regulation of 11beta-hydroxysteroid dehydrogenase type 1 gene expression in human ovarian surface epithelial cells by interleukin-1." Hum Reprod **17**(9): 2300-6.

Yousef, G. M. and E. P. Diamandis (2002). "Human tissue kallikreins: a new enzymatic cascade pathway?" Biol Chem **383**(7-8): 1045-57.

Zand, L., F. Qiang, et al. (2003). "Differential effects of cellular fibronectin and plasma fibronectin on ovarian cancer cell adhesion, migration, and invasion." In Vitro Cell Dev Biol Anim **39**(3-4): 178-82.

Zeisler, H., C. Tempfer, et al. (1998). "Serum interleukin 1 in ovarian cancer patients." Eur J Cancer **34**(6): 931-3.

Zhao, Y., V. R. Agarwal, et al. (1996). "Estrogen biosynthesis proximal to a breast tumor is stimulated by PGE2 via cyclic AMP, leading to activation of promoter II of the CYP19 (aromatase) gene." Endocrinology **137**(12): 5739-42.

Zhao, Y., H. Rong, et al. (1995). "Expression and selective cellular localization of granulocyte-macrophage colony-stimulating factor (GM-CSF) and GM-CSF alpha and beta receptor messenger ribonucleic acid and protein in human ovarian tissue." Biol Reprod **53**(4): 923-30.

Zheng, H., J. J. Kavanagh, et al. (2007). "Hormonal therapy in ovarian cancer." Int J Gynecol Cancer **17**(2): 325-38.

Zheng, W., J. J. Lu, et al. (2000). "Ovarian epithelial tumor growth promotion by follicle-stimulating hormone and inhibition of the effect by luteinizing hormone." Gynecol Oncol **76**(1): 80-8.

Ziltener, H. J., S. Maines-Bandiera, et al. (1993). "Secretion of bioactive interleukin-1, interleukin-6, and colony-stimulating factors by human ovarian surface epithelium." Biol Reprod **49**(3): 635-41.

Zygmunt, M., F. Herr, et al. (2002). "Characterization of human chorionic gonadotropin as a novel angiogenic factor." J Clin Endocrinol Metab **87**(11): 5290-6.

Presentations

Poster presentations

• 16-19 March 2009, Harrogate, UK: Society for Endocrinology 2009 meeting

IL-1α regulation of Steroid Sulphatase (STS) enzyme activity and 17 β-hydroxysteroid dehydrogenase 5(17β-HSD5) gene expression in normal human ovarian surface epithelium (OSE), epithelial ovarian cancer (EOC) and cell lines

• 15-18 March 2010, Manchester, UK: Society for Endocrinology 2010 meeting

Expression and regulation of oestrogen sulfotransferase (EST) in human ovarian surface epithelium (OSE) and epithelial ovarian cancer (EOC)

• 21-24 September, 2010, Edinburgh, UK: 14th International Congress for Hormonal Steroids and Hormonal Cancer

Natural and hormone replacement therapy (HRT)-derived oestrogen regulation of Insulin-like growth factor binding protein 3 (IGFBP3) and Fibronectin1 (FN1) in epithelial ovarian cancer (EOC)

Oral presentation

• 12-13 September, 2009, Cambridge, UK: 10th National Ovarian Workshop

Might steroid sulphatase (STS) be a target of ovarian cancer treatment?